Exhibit 71

REVIEW

The Role of the Mediators of Inflammation in Cancer Development

José Veríssimo Fernandes · Ricardo Ney Oliveira Cobucci ·
Carlos André Nunes Jatobá · Thales Allyrio Araújo de Medeiros Fernandes ·
Judson Welber Veríssimo de Azevedo · Josélio Maria Galvão de Araújo

Abstract Epigenetic disorders such as point mutations in cellular tumor suppressor genes, DNA methylation and post-translational modifications are needed to transformation of normal cells into cancer cells. These events result in alterations in critical pathways responsible for maintaining the normal cellular homeostasis, triggering to an inflammatory response which can lead the development of cancer. The inflammatory response is a universal defense mechanism activated in response to an injury tissue, of any nature, that involves both innate and adaptive immune responses, through the collective action of a variety of soluble mediators. Many inflammatory signaling pathways are activated in several types of cancer, linking chronic inflammation to tumorigenesis process. Thus, Inflammatory responses play decisive roles at different stages of tumor development, including initiation, promotion, growth, invasion, and metastasis, affecting also the immune surveillance. Immune cells that infiltrate tumors engage in an extensive and dynamic crosstalk with cancer cells, and some of the molecular events that mediate this dialog have been revealed. A range of inflammation mediators, including cytokines, chemokines, free radicals, prostaglandins, growth and transcription factors, microRNAs, and enzymes as, cyclooxygenase and matrix metalloproteinase, collectively acts to create a favorable microenvironment for the development of tumors. In this review are presented the main mediators of the inflammatory response and discussed the likely mechanisms through which, they interact with each other to create a condition favorable to development of cancer.

Keywords Inflammation and cancer · Inflammation mediators · Mechanisms of tumorigenesis

J. V. Fernandes · J. M. G. de Araújo
Post Graduate Program in Biological Sciences, Federal University of Rio Grande do Norte, Natal, RN, Brazil

J. V. Fernandes (✉) · J. M. G. de Araújo
Department of Microbiology and Parasitology, Federal University of Rio Grande do Norte, Campus Universitário, 59072 910 Natal, RN, Brazil
e mail: joseverissimo1951@gmail.com

R. N. O. Cobucci
Maternidade Escola Januário Cicco, Federal University of Rio Grande do Norte, Natal, RN, Brazil

C. A. N. Jatobá
Department of Pathology, Federal University of Rio Grande do Norte, Natal, RN, Brazil

T. A. A. de Medeiros Fernandes
Department of Biomedical Sciences, University of Rio Grande do Norte State, Mossoró, RN, Brazil

J. W. V. de Azevedo
Hospital Universitário, Federal University of Triângulo Mineiro, Mina Gerais, Brazil

Abbreviations

AA	Arachidonic acid
AP-1	Activator protein 1
APC	Antigen-presenting cell
cAMP	Cyclic AMP
CCL	Chemokine (C-C motif) ligand
CD	Cluster of differentiation
cHL	Classical Hodgkin lymphoma
CLRs	C-type lectin receptors
COX	Cyclooxygenase
CRC	Colorectal cancer
CXC	Chemokine receptors
DAMPs	Damage-associated molecular patterns
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
FOXP3	Forkhead box P3

 Springer

528   J.V. Fernandes et al.

GPCRs   G protein-coupled
HPV   Human papillomavirus
ICC   Invasive cervical cancer
IFN   Interferon
IL   Interleukin
MHC   Major histocompatibility complex
miRNAs   MicroRNAs
MM   Multiple myeloma
MMPs   Enzymes matrix metalloproteinase matrix
NF-κB   Nuclear factor kappa-light-chain-enhancer of activated B cells
NK   Natural killer cell
NLRs   NOD-like receptors
NO   Nitric oxide
NSAIDs   Non-steroidal anti-inflammatory drugs
p53   Tumor protein p53
PAMPs   Pathogen-associated molecular patterns
PGs   Prostaglandins
PRRs   Pattern recognition receptors
PTGER   Prostaglandin receptor
PTGES   Terminal prostaglandin synthase enzyme
PubMed   US National Library of Medicine
RLRs   RIG-like receptors
ROS   Reactive oxygen species
STAT   Signal transducers and activators of transcription
TCR   T Cell Receptor
TGF   Transforming growth factor
Th cells   T helper cells
TLRs   Toll-like receptors
TNF   Tumor necrosis factor
Tregs   Regulatory T cells
Txs   Thromboxanes

Introduction

The primary functions of inflammation are rapidly destroying or isolating the underlying source of the disturbance and then restoring homeostasis so that, being regulated properly, behaves as an adaptive mechanism. One indication of this is the fact that humans with primary genetic defects in the components of inflammation have increased risk of serious infections. A similar phenomenon was observed in animals with defects in genes encoding pro-inflammatory cytokines [1]. Moreover, immunologically relevant genes whose dysfunction leads to spontaneous inflammation are not expressed under normal conditions, suggesting that the inflammatory response is suppressed to maintain health since its deregulation can have devastating effects for the host, resulting in collateral damage and pathology [2]. Thus, despite being a designed response to eliminate pathogens and other agents harmful to the host, the inflammation when deregulated or

inappropriately maintained has the potential to cause injury, necrosis, and malignant transformation [3].

Much evidence supports the hypothesis that inflammation participates in providing conditions that lead to cancer. An unresolved inflammation due to any failure in precise control of the immune response can lead to alterations in expression of cancer-related genes and posttranslational modification in cellular proteins involved in the cell cycle, DNA repair, and apoptosis favoring the development of cancer [4]. Currently, it is well established that chronic inflammation is strongly associated with several human cancers, since it leads to the release of pro-inflammatory cytokines, and other immunomodulatory, creating a favorable microenvironment for tumor progression and metastasis [5].

The inflammation generates oxidative stress, which in turn increases inflammation, so that the two are common denominators in carcinogenesis. Oxidative stress generates reactive oxygen species (ROS) that causes DNA damage and activates signaling pathways that deregulate the cell cycle and hence increase the risk of development of cancers. There is a cross-talk between these two mediators, where ROS and inflammation potentiate each other to ultimately cause cancer [6]. Thus, the inflammatory response plays key roles at different stages of tumor development, besides affecting immune surveillance. Immune cells that infiltrate into tumors establish a cross-talk with cancer cells to orchestrate interactions between different mechanisms, which together can lead to the formation of tumors. This review presents a discussion of some mediators of inflammation and the molecular events through which communication is established between immune and tumor cells, as key mechanisms regulating the effects of inflammation and immunity on tumor development.

The literature review was conducted in the electronic databases PubMed (National Institutes of Health), Scopus (Elsevier), and Web of Knowledge (Thomson Reuters), using the following keywords: carcinogenesis, Inflammation and cancer. The databases retrieved hundreds of articles, and we selected those that we thought to be most relevant to our purpose.

Mediators Involved in the Inflammation and Carcinogenesis

The Infections and chronic inflammation contribute to about 1 in 4 of all cancer cases. Mediators of the inflammatory response, such as: cytokines, chemokines, free radicals, prostaglandins, growth factors and enzymes as cyclooxygenase (COX) and matrix metalloproteinase, can induce genetic and epigenetic changes, that result in alterations in critical pathways responsible for maintaining the normal cellular homeostasis and can leading to the development and progression to cancer [7–9].

Cytokines and chemokines are involved in many aspects of growth, differentiation and cell activation. Table 1 summarizes the actions of the main cytokines that play some role in the activation or regulation of the inflammatory response and that contribute in some way to the process of tumorigenesis.

Chemokines are key players of the cancer-related inflammation, whereas their respective receptors and ligands are the downstream genetic events that cause neoplastic transformation and which are abundantly expressed in chronic inflammation, increasing susceptibility to cancer. The components of the chemokine system affect different routes of tumor progression, including leukocyte recruitment, neo-angiogenesis, proliferation, survival, invasion, and metastasis of tumor cells. Preclinical and clinical trials indicate that the intervention in the chemokine system can be a valuable tool for the development of future therapeutic strategies against cancer [35].

It has been shown that the CXCR2 chemokine receptor and its ligands promote angiogenesis and leukocyte infiltration in the tumor microenvironment. In the acidic and hypoxic conditions of the tumor microenvironment, up-regulating the expression of CXCR4 creates a gradient prepared by CXCL12 for migration of tumor-associated fibroblasts (CAF). The axis CXCL12-CXCR4 facilitates metastasis to distant organs and the CCL21-CCR7 chemokine ligand-receptor pair favors metastasis to lymph nodes. These two chemokine ligand-receptor systems are common key mediators of tumor cell metastasis for several malignancies [36].

It has been shown that cancer cells secrete, or induce fibroblasts to secrete the chemokine CCL5, which acts in an autocrine or paracrine manner on tumor cells, which express their receptor (CCR5). This promotes the proliferation of these cells and recruitment of T-reg cells and monocytes to induce activation of osteoclasts and bone metastases, by inducing neoangiogenesis, and to facilitate the spread of tumor cells for distant organs. It is believed that CCL5, produced by cells of classical Hodgkin lymphoma (cHL), may represent an autocrine growth factor of the tumor cells by creating a microenvironment conducive to tumor progression, whereas CCL5 secreted by T cells or fibroblasts may represent a paracrine growth factor. TCD4+ cells expressing CD40L increase the secretion of CCL5 by cHL cells and induce secreting CCL5 by fibroblasts, which promote the recruitment of activated fibroblasts by cHL cells, which in turn recruit T-reg cells, eosinophils, and mast cells [35].

It has been observed that CXCL8, a chemokine of the CXC family, exerts its effects through signaling two G-coupled receptors, CXCR1 and CXCR2 protein. Elevated CXCL8 signaling - CXCR1 / 2 within the tumor microenvironment of various types of human cancers promotes tumor progression through the activation of signaling pathways involved in activation of proliferation, survival, angiogenesis, migration, and cell invasion, through transactivation of the epidermal growth factor receptor (EGFR) [5].

The Role of Transcription Factors NF-κB

The NF-κB family of transcription factors has been recognized as a crucial player in many steps of cancer including initiation and progression, cooperating with multiple other signaling molecules and pathways. This action is mediated by other transcription factors such as STAT3 and p53 or the ETS-related gene ERG, which directly interacts with NF-κB subunits or affects NF-κB target genes. Crosstalk can also occur through different kinases, such as GSK3-β, p38, or PI3K, which modulate NF-κB transcriptional activity or affect upstream signaling pathways. Other classes of molecules that can also act in the integration of these mechanisms involving NF-κB are reactive oxygen species and miRNAs [37].

It is well known that NF-κB regulate the expression of numerous cytokines and adhesion molecules which are critical elements involved in the regulation of immune responses [38]. Furthermore, it coordinates the central signaling pathways of activation of the innate and adaptive immune responses, and that STAT3 regulates the expression of various genes in response to cellular stimuli, playing a key role in cell growth and apoptosis. It has been shown that STAT3 is constitutively activated in many human cancers, including gastric cancer and plays crucial roles in modulating proliferation and survival, cancer cells as well as creating a favorable microenvironment to the formation of metastasis [39].

The activation and interaction between STAT3 and NF-κB have been widely investigated in human cancers such as colon, stomach, and liver cancers. It has been shown that the interaction between these two transcription factors play a vital role in controlling the communication between inflammatory cells and cancerous cells. NF-κB and STAT3 are the main two factors that control the capacity of pre-neoplastic and malignant tumor cells to resist immune surveillance by regulating apoptosis, angiogenesis, and tumor invasion. The understanding of the molecular mechanisms of NF-κB and STAT3 cooperation in cancer development will provide opportunities for the design of new chemo-preventive and chemotherapeutic approaches [40].

The Role of Matrix Metalloproteinase and Cyclooxygenases in the Carcinogenesis

The matrix metalloproteinases (MMPs) are members of the metzincin group of proteases, and constitute a family of zinc-dependent proteolytic enzymes that degrade various components of the extracellular matrix (ECM). Due to their broad spectrum of substrate specificity, MMPs contribute to the homeostasis of many tissues and participate in diverse physiological processes, such as bone remodeling, angiogenesis, wound healing, and immunity. However, the unregulated

Table 1 The role of some cytokines in cancer

Cytokine	Role in cancer development	Ref.
Interleukin 1β (IL 1β)	Supression of p53 expression; Cancerous epithelial cells uses IL 1β as a communication factor instructing stromal fibroblasts, whose expression of p53 was suppressed, creating an inflammatory microenvironment and protumorigenic	[10]
Tumor necrosis factor α (TNF α)	Creation of a tumor microenvironment that stimulates the growth and survival of tumor cells through the induction of gene encoding NF κB dependent antiapoptotic molecules. Furthermore, It cause inflammatory cell infiltration in tumors and promotes angiogenesis, invasion and migration of tumor cell, and suppress cytotoxic T lymphocytes and activated macrophages. TNF α also contributes to the initiation of tumors through the stimulation of production of genotoxic molecules such as nitric oxide (NO) and ROS, which may cause DNA mutations	[11 13]
Transforming growth factor β (TGF β)	TGF β is essentially an inhibitory cytokine with an anti inflammatory and immunosuppressive action, and has a central role in the proliferation and function of Treg cells. Changes in its signaling pathways are often observed in human cancer. These alterations attenuate the TGF β tumor suppressive effects, promoting tumor progression and metastasis. The carcinoma often secrete this cytokine in excess, resulting in increased epithelial mesenchimal transition with tissue invasion and metastasis.	[14 16]
Interleukin 6 (IL 6)	Stimulation of angiogenesis, promotion of cell proliferation and increased survival of malignant cells, besides inhibit the apoptosis of cancer cells. Clinical studies have shown that high serum levels of IL 6 are associated with advanced stages of various cancers.	[17, 18]
Interleukin 10 (IL 10)	Inhibition of IFN γ production by Th1 cells as well as production of inflammatory cytokine, including TNF α, IL 6, and IL 12. Therefore, it is involved in the inhibition of tumor development and progression. However, depending on the context in witch it acts, this cytokine can have action against or favorable to development of tumor. Its presence in the inflammatory microenvironment of the tumor can eliminate the anticancer action of the Th1 response. On the other hand, IL 10 and Tregs also suppress the activity of Th17, which is associated with poor prognosis in several types of cancer.	[19 25]
Interleukin 17 (IL 17)	Induction of many proinflamatory mediators, including TNF α, IL 1β, and IL 6, suggesting a role in locating and amplifying the inflammation. Besides, several studies have shown large amounts of Th17 cells infiltrated in tumors and high levels of expression of IL 17 in the serum of patients with several types of tumors, suggesting an important role in the tumorigenesis. The Th17/Treg balance was also broken in the periphelical blood of cervical cancer patients.	[26 28]
Interleukin 12 (IL 12)	IL 12 has a protective activity against cancer, acting to prevent initiation, growth, and metastasis of tumors. It stimulates the cytotoxic activity and production of IFN γ and TNF α from NK and TCD8 cells, promoting a TH1 immune response, besides an antiangiogenic function. Recently, it has become evident the balance between IL 12 and IL 23 (a promoter of Th17 immune response) is important in the carcinogenesis process.	[29, 30]

Table 1 (continued)

Cytokine	Role in cancer development	Ref.
Interleukin 18 (IL 18)	IL 18 acts in synergy with IL 12 to induces Th1 immune response against cancer. The systemic administration of IL 18 has been chown to have significant antitumor activity in several preclinical animal models. However, its expression and secretion has been observed in several types of immune cells promoting cancer. Its levels has also been elevated in patients with squamous cell carcinoma of the skin.	[31 34]

activity of MMPs leads to pathological conditions such as arthritis, inflammation, and cancer [41, 42].

They are key regulators of ECM and basement membranes, contributing to the development and progression of human malignant tumors due to their interaction with the receptors for growth factors, cytokines, chemokines, cell adhesion molecules, apoptotic ligands, and angiogenic factors [43, 44].

There are several different types of MMPs, including MMP-1, MMP −2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-12, MP-13, and MT1-MMP, which are stimulated and activated by various mechanisms in vascular tissues. Once activated, MMPs degrade ECM proteins and other related signaling molecules, promoting abnormal angiogenesis and remodeling of vascular tissue, and facilitating recruitment of stem / progenitor cells, endothelial cells (ECs), and vascular smooth muscle cells (VSMCs). The changes in the behavior of these cells contribute to the pathogenesis of various disorders [43].

MMPs regulate inflammation by substrate processing of a range of novel substrates including chemokines, growth factors, receptors, binding proteins, proteases, protease inhibitors, and extra-and intracellular multifunctional proteins [45]. MMP-1 and MMP-13 are collagenases that degrade ECM, especially the collagens of type I, II, and III, which are the main components of the interstitial stroma. In colorectal cancer (CRC), the expression of MMP-1 is correlated with a more advanced stage of disease and with poor prognosis. It has been observed that the level of invasion of the lymph nodes by metastasis in CRC were associated with elevated levels of MMP-1. It has been shown that the expression of MMP-13 may be related to tumor biological aggressiveness and used to aid in predicting patient's poor prognosis. In fact, the expression MMP-13 expression was correlated with the decreased survival of patients with CRC [46].

MMP-2 and MMP-9 are gelatinases whose main substrate is type IV collagen and gelatin, but they also have proteolytic activity against other extracellular matrix molecules. Higher levels of expression of these enzymes were found in the plasma of patients with CRC that have metastasis in lymph nodes compared with those without lymph node metastases. MMP-7 is a matrilysin whose expression has been observed in about 80 % of all cases of CRC, and its serum levels are associated with the progression of CRC and decreased survival rate. MMP-7 promotes cancer invasion through cleavage of ECM proteins and activates other MMPs, including proMMP-2 and proMMP-9, to promote invasion of cancer cells. MMP-12 is a metalloelastase expressed predominantly in the macrophage, and it is able to degrade many different substrates and seems to have a protective function in CRC, since its inhibition was considered potentially harmful to the patient with this pathology [44].

On the other hand, the cyclooxygenases are enzymes that convert free arachidonic acid (AA) into prostanoids, including prostaglandins (PGs) and thromboxanes (Txs). There are two isoforms of COX designated, COX-1 and COX-2, being COX-2 the most strongly linked to development and progression of cancer [47, 48]. High expression levels of COX-2 are found in the tissue of colorectal cancer (CRC) and are associated with less survival of patients with CRC [49]. The clinical and epidemiological studies and animal experiments indicate that non-steroidal anti-inflammatory drugs (NSAIDs) are among the most promising chemopreventive agents for this disease. The NSAIDs exert their anti-inflammatory and antitumor effects mainly by inhibiting the action of COX-2, leading to reduced production of prostaglandins [50].

In cells of invasive cervical cancer (ICC), E5, E6, and E7 HPV 16 oncogenes were able to induce the COX/ prostaglandin inflammatory axis by increasing the expression of the COX-2 gene [9]. This suggests a direct link between HPV oncogene and activation of an inflammatory response, a potent factor in promoting cancer. Thus, although the initial HPV infection is not associated with inflammation, it is believed that, after integration of the virus into the cell genome, viral persistence occurs, followed by malignant transformation of the infected cell. This occurs due to the activation of inflammatory pathways such as COX-prostaglandin promoting an infiltration of inflammatory and immune cells, creating a favorable microenvironment for tumor progression [51].

Both COX 1 and 2 are significantly represented in cells of ICC, and the products of HPV oncogene and of the PGE2 gene can regulate the expression of the prostaglandin receptor (PTGER) [52]. Furthermore, it was demonstrated that E5 of

the HPV16 protein regulates the expression of PTGER4 in cells of ICC in a way that is dependent on PGE2 production of cyclic AMP (cAMP). This suggests that increased levels of PGE2 on ICC may regulate the function of neoplastic cells in an autocrine or paracrine manner, through the expression of high levels of PTGER2 and PTGER4 prostaglandin receptors [53].

The Role of microRNAs in the Carcinogenesis

MicroRNAs (miRNAs) are small noncoding single-stranded RNAs, which are highly conserved during evolution, and controls the gene expression by degrading the corresponding mRNA, destabilizing and/or inhibition their translation [54]. They have been implicated in the regulation of almost all aspects of cellular functions, including the immune responses, innate and adaptive. miRNAs are involved in many types of inflammatory responses and have a significant impact on the magnitude of the responses. Furthermore, they participate of many regulatory networks of genes whose dysfunctions are associated with human diseases such as cancer [55, 56].

The expression of miRNAs is tightly controlled both spatially and temporally. Although some of them may function as tumor suppressors, the aberrant expression of these molecules has been correlated with various types of human cancers [57]. Besides, several miRNAs are involved in many types of inflammatory response. This is done in two main ways: by affecting development of subpopulations of inflammatory cells such as Th2 and Th17, or by setting the level of immune cell function, e.g., controlling the amount of cytokine produced by DCs [58].

Some miRNAs are expressed in activated T lymphocytes, and each miRNA represses its specific targets, which are often transcription factors specific for a given cell line. This may determines the type of inflammatory T cells produced during inflammation. Specific miRNAs, such as miR-155 and miR-146a, expressed in inflammatory cells, have as targets signaling proteins that regulate the intensity of the inflammatory signal. Ideally, the signaling results in a transient inflammatory response that eliminates the infection without harming the host. The lack of certain miRNAs, such as miR-155, can reduce the magnitude of the immune response, resulting in immunodeficiency. On the other hand, the constant overexpression of miR-155 or deletion of miR-146a can cause a chronic inflammatory condition in which inflammation is not resolved [59].

The expression of miR-21, miR-155, and miR125b is controlled by an undetermined amount of immune signals, the most prominent being TLR, TNF-α, and other cytokines that bind the functions of these miRNAs with inflammatory events [60]. The inflammation modulates the expression of microRNAs that influence the production of several tumor-

related messenger RNAs or proteins. These molecular events induced by chronic inflammation contributes to alter important pathways involved in normal cellular function, and hence strengthen the role of inflammation in cancer development [61]. miR-21 is unregulated, both in vitro and in vivo, by oncogenes RAS or SRC, the most frequently activated in human cancers [62].

Among the mechanisms used by miRNAs to promote the initiation and progression of tumors are those that affect the modulation of TLR, cytokines, and their signaling pathways, they also play an important role in the development of cancers associated with infectious agents. The infections with various pathogens induce changes in the expression of miRNAs functionally related to the mounting of the innate immune response. Thus, they are involved in the regulation of the survival and proliferation of immunocompetent cells responsible for the control of infections. The miRNAs miR-21, miR-125, and miR-155 are the most frequently expressed during infection and therefore have a potential role in carcinogenesis induced by infectious agents. It has been shown that overexpression of miR-21 and miR-182 is associated with carcinogenesis associated with HPV with high oncogenic potential [60, 63].

A recent study identified one inflammatory pathway mediated by microRNA that is epigenetically repressed in breast cancers. A high-throughput screen for signal transducer and activator of transcription 3 (STAT3)–regulated microRNAs revealed the microRNA miR-146b as a direct STAT3 target in mammary epithelial cells, but DNA methylation in its promoter area suppressed miR-146b expression in cancer cells. It was observed that deregulated expression of miR-146a and miR-155, facilitates the development of proinflammatory phenotype of Tregs via increased STAT1 activation [64]. Overexpression of miR-146b suppresses NF-kB in an IL-6-dependent manner. The subsequent STAT3 activation decreased invasiveness phenotype in breast cancer cells [65, 66]. It has been proposed that carcinogenesis induced by inflammatory response triggered by miRNA, in colon cancer is related to dysregulation of colon cells and leukocytes, with impact on proteins involved in the PI3K/Atk signaling pathway, thereby contributing to cancer cell proliferation and tumor growth [67].

Conclusions

Chronic inflammation arising of infections or of autoimmune disease precedes development of tumors, suggesting that inflammatory response plays an important role in the tumorigenesis process. Studies show that chronic inflammation can contribute to initiation, promotion, growth, and invasion of tumors, through of oncogenes activation, induction of mutations, loss of the mechanisms of cell cycle control, and of DNA repair, generating a genomic instability which, together

with angiogenesis and tissue remodeling, contributes to development about 1 in 4 cases, of cancer. The mediators of inflammatory response coordinates the central signaling pathways of activation of the innate and adaptive immune responses, and affect various aspects of inflammation, by activating involved genes in survival and proliferation of cells. Also promotes processing the extracellular matrix proteins and other related signaling molecules, causing abnormal angiogenesis and remodeling of vascular tissue, facilitating recruitment and activation or suppression cells of the immune system. Thus, a large variety of inflammatory mediators act together through a complex network of communication through which, they interact with each other's, of synergistic or antagonistic way, to break the cellular homeostasis, creating favorable conditions for initiation, progression and invasion of tumors. Understanding the mechanisms involved in activation, migration and infiltration of immune cells into tumors, as well as the role of a range of mediators of inflammation in the crosstalk of the immune cells with cancer cells, and the molecular events that mediate this dialog, is of great importance to find ways of intervene in this complex network of events, in order of prevent or interrupt the process of tumorigenesis.

References

1. Martinon F, Mayor A, Tschopp J (2009) The inflammasomes: guardians of the body. Annu Rev Immunol 27:229 265

2. Ashley NT, Weil ZM, Nelson RJ (2012) Inflammation: mechanisms, costs, and natural variation. Annu Rev Ecol Evol Syst 43:385 406

3. Bermejo Martin JF, Martín Loeches I, Bosinger S (2014) Inflammation and infection in critical care medicine. Mediat Inflamm 2014:456256. doi:10.1155/2014/456256

4. Eiró N, Vizoso FJ (2012) Inflammation and cancer. World J Gastrointest Surg 4(3):62 72

5. Campbell LM, Maxwell PJ, Waugh DJ (2013) Rationale and means to target pro inflammatory interleukin 8 (CXCL8) signaling in cancer. Pharmaceuticals 6(8):929 959

6. Kaushal N, Kudva AK (2013) Oxidative stress and inflammation: the lesser of two evils in carcinogenesis. PostDoc J 1(2):89 101

7. Parks WC, Wilson CL, López Boado YS (2004) Matrix metalloproteinases as modulators of inflammation and innate immunity. Nat Rev Immunol 4(8):617 629

8. Hussain SP, Harris CC (2007) Inflammation and cancer: an ancient link with novel potentials. Int J Cancer 121(11):2373 2380

9. Adefuye A, Sales K (2012) Regulation of inflammatory pathways in cancer and infectious disease of the cervix. Scientifica (Cairo) 2012:548150

10. Allavena P, Germano G, Marchesi F, Mantovani A (2011) Chemokines in cancer related inflammation. Exp Cell Res 317(5):664 673

11. Raman D, Baugher PJ, Thu YM, Richmond A (2007) Role of chemokines in tumor growth. Cancer Lett 256(2):137 165

12. Hoesel B, Schmid JA (2013) The complexity of NF κB signaling in inflammation and cancer. Mol Cancer 12:86. doi:10.1186/1476 4598 12 86

13. Liang Y, Zhou Y, Shen P (2004) NF kappaB and its regulation on the immune system. Cell Mol Immunol 1(5):343 350

14. Wu LJ, Li HX, Luo XT et al (2014) STAT3 activation in tumor cell free lymph nodes predicts a poor prognosis for gastric cancer. Int J Clin Exp Pathol 7(3):1140 1146

15. Fan Y, Mao R, Yang J (2013) NF κB and STAT3 signaling pathways collaboratively link inflammation to cancer. Protein Cell 4(3):176 185

16. Gomis Rüth FX (2009) Catalytic domain architecture of metzincin metalloproteases. J Biol Chem 284(23):15353 15357

17. Löffek S, Schilling O, Franzke CW (2011) Biological role of matrix metalloproteinases: a critical balance. Eur Respir J 38:191 208

18. Walter L, Harper C, Gar P (2013) Role of matrix metalloproteinases in inflammation/colitis associated colon cancer. Immuno Gastroenterology 2(1):22 28

19. Chen Q, Jin M, Yang F, Zhu J, Xiao Q, Zhang L (2013) Matrix metalloproteinases: inflammatory regulators of cell behaviors in vascular formation and remodeling. Mediat Inflamm 2013:928315. doi:10.1155/2013/928315

20. Butler GS (2000) Overall CM (2013) Matrix metalloproteinase processing of signaling molecules to regulate inflammation. Periodontol 63(1):123 148

21. Said AH, Raufman JP, Xie G (2014) The role of matrix metalloproteinases in colorectal cancer. Cancers 6(1):366 375

22. Zyada MM, Shamaa AA (2008) Is collagenase 3 (MMP 13) expression in chondrosarcoma of the jaws a true marker for tumor aggressiveness? Diagn Pathol 3:26. doi:10.1186/1746 1596 3 26

23. Zha S, Yegnasubramanian V, Nelson WG, Isaacs WB, De Marzo AM (2004) Cyclooxygenases in cancer: progress and perspective. Cancer Lett 215(1):1 20

24. Cathcart MC, O'Byrne KJ, Reynolds JV, O'Sullivan J, Pidgeon GP (2012) COX derived prostanoid pathways in gastrointestinal cancer development and progression: novel targets for prevention and intervention. Biochim Biophys Acta 1825(1):49 63

25. Asting AG, Carén H, Andersson M, Lönnroth C, Lagerstedt K, Lundholm K (2011) COX 2 gene expression in colon cancer tissue related to regulating factors and promoter methylation status. BMC Cancer 11:238. doi:10.1186/1471 2407 11 238

26. Wang D, Dubois RN (2010) The Role of COX 2 in intestinal inflammation and colorectal cancer. Oncogene 29(6):781 788

27. Sharkey DJ, Tremellen KP, Jasper MJ, Gemzell Danielsson K, Robertson SA (2012) Seminal fluid induces leukocyte recruitment and cytokine and chemokine mRNA expression in the human cervix after coitus. J Immunol 188(5):2445 2454

28. Sales KJ, Katz AA, Howard B et al (2002) Cyclooxygenase 1 is up regulated in cervical carcinomas: autocrine/paracrine regulation of cyclooxygenase 2, prostaglandin e receptors, and angiogenic factors by cyclooxygenase 1. Cancer Res 62:424 432

29. zur Hausen H (2009) Papillomaviruses in the causation of human cancers   a brief historical account. Virology 384(2):260 265

30. Ahmad J, Hasnain SE, Siddiqui MA, Ahamed M, Musarrat J, Al Khedhairy AA (2013) MicroRNA in carcinogenesis & cancer diagnostics: a new paradigm. Indian J Med Res 137(4):680 694

31. Croce CM (2009) Causes and consequences of microRNA dysregulation in cancer. Nat Rev Genet 10:704 714

32. Ostermann E, Laventie JR, Pfeffer S, Bahram S, Sprauel PS, Georgel P (2013) MicroRNAs: Fine turners of type I interferon dependent inflammatory responses. Curr Trends Immunol 14:35 44

33. Tili E, Michaille JJ, Croce CM (2013) MicroRNAs play a central role in molecular dysfunctions linking inflammation with cancer. Immunol Rev 253(1):167 184

34. Shivdasani RA (2006) MicroRNAs: regulators of gene expression and cell differentiation. Blood 108(12):3646 3653

35. Yeung ML, Jeang KT (2011) MicroRNAs and cancer therapeutics. Pharm Res 28(12):3043 3049

36. O'Connell RM, Rao DS, Baltimore D (2012) MicroRNA regulation of inflammatory responses. Annu Rev Immunol 30:295 312

37. Chiba T, Marusawa H, Ushijima T (2012) Inflammation associated cancer development in digestive organs: mechanisms and roles for genetic and epigenetic modulation. Gastroenterology 143(3):550 563

38. Schetter AJ, Okayama H, Harris CC (2012) The role of microRNAs in colorectal cancer. Cancer J 18:244 252

39. Tang T, Wong HK, Gu W et al (2013) MicroRNA 182 plays an onco miRNA role in cervical cancer. Gynecol Oncol 129(1):199 208

40. Zhou Q, Haupt S, Kreuzer JT et al (2014) Decreased expression of miR 146a and miR 155 contributes to an abnormal Treg phenotype in patients with rheumatoid arthritis. Ann Rheum Dis. doi:10.1136/annrheumdis 2013 204377

41. Iliopoulos D (2014) MicroRNA circuits regulate the cancer inflammation link. Sci Signal 7(318):pe8. doi:10.1126/scisignal.2005053

42. Josse C, Bouznad N, Geurts P et al (2014) Identification of a microRNA landscape targeting the PI3K/Akt signaling pathway in inflammation induced colorectal carcinogenesis. Am J Physiol Gastrointest Liver Physiol 306(3):G229 G243

43. Schauer IG, Zhang J, Xing Z, Guo X, Mercado Uribe I, Sood AK, Huang P, Liu J (2013) Interleukin 1β promotes ovarian tumorigene sis through a p53/NF κB mediated inflammatory response in stromal fibroblasts. Neoplasia 15(4):409 420

44. Lou JL, Maeda S, Hsu LC, Yagita H, Karin M (2004) Inhibition of NF kappaB in cancer cells converts inflammation induced tumor growth mediated by TNF α to TRAIL mediated tumor regression. Cancer Cell 6(3):297 305

45. Waters JP, Pober JS, Bradley JR (2013) Tumour necrosis factor and cancer. J Pathol 230(3):241 248

46. Hussain SP, Hofseth LJ, Harris CC (2003) Radical causes of cancer. Nat Rev Cancer 3(4):276 285

47. Lebrun JJ (2012) The dual role of TGF β in human cancer: from tumor suppression to cancer metastasis. ISRN Mol Biol 2012:381428. doi:10.5402/2012/ 381428

48. Elliott RL, Blobe GC (2005) Role of transforming growth factor beta in human cancer. J Clin Oncol 23(9):2078 2093

49. Chen W, Konkel JE (2010) TGF beta and 'adaptive' Foxp3(+) regu latory T cells. J Mol Cell Biol 2(1):30 36

50. Visperas A, Do JS, Bulek K, Li X, Min B (2014) IL 27, targeting antigen presenting cells, promotes Th17 differentiation and colitis in mice. Mucosal Immunol 7(3):625 633

51. Guo Y, Xu F, Lu T, Duan Z, Zhang Z (2012) Interleukin 6 signaling pathway in targeted therapy for cancer. Cancer Treat Rev 38(7):904 910

52. Vicari AP, Trinchieri G (2004) Interleukin 10 in viral diseases and cancer: exiting the labyrinth? Immunol Rev 202:223 236

53. Mocelin S, Marincola FM, Young HA (2005) Interleukin 10 and the immune response against cancer: a counterpoint. J Leukoc Biol 78:1043 1051

54. Driessler F, Venstrom K, Sabat R, Asadullah K, Schottelius AJ (2004) Molecular mechanisms of interleukin 10 mediated inhibition of NF kappaB activity: a role for p50. Clin Exp Immunol 135(1):64 73

55. Pattison MJ, MacKenzie KF, Arthur JS (2012) Inhibition of JAKs in macrophages increases lipopolysaccharide induced cytokine produc tion by blocking IL 10 mediated feedback. J Immunol 189(6):2784 2792

56. Sato T, Terai M, Tamura Y, Alexeev V, Mastrangelo MJ, Selvan SR (2011) Interleukin 10 in the tumor microenvironment: a target for anticancer immunotherapy. Immunol Res 51(2 3):170 82

57. Cope A, Le Friec G, Cardone J, Kemper C (2011) The Th1 life cycle: molecular control of IFN γ to IL 10 switching. Trends Immunol 32(6):278 286

58. Stewart CA, Metheny H, Iida N et al (2013) Interferon dependent IL 10 production by Tregs limits tumor Th17 inflammation. J Clin Invest 123(11):4859 4874

59. Shigehara K, Shijubo N, Ohmichi M et al (2001) IL 12 and IL 18 are increased and stimulate IFN γ production in sarcoid lungs. J Immunol 166(1):642 649

60. Wong JL, Berk E, Edwards RP, Kalinski P (2013) IL 18 primed helper NK cells collaborate with dendritic cells to promote recruit ment of effector CD8+ T cells to the tumor microenvironment. Cancer Res 73(15):4653 4662

61. Srivastava S, Salim N, Robertson MJ (2010) Interleukin 18: biology and role in the immunotherapy of cancer. Curr Med Chem 17(29):3353 3357

62. Palma G, Barbieri A, Bimonte S, Palla M, Zappavigna S, Caraglia M, Ascierto PA, Ciliberto G, Arra C (2013) Interleukin 18: friend or foe in cancer. Biochim Biophys Acta 1836:296 303

63. Miossec P (2009) IL 17 and Th17 cells in human inflammatory dis eases. Microbes Infect 11(5):625 630

64. Xiang T, Long H, He L, Han X, Lin K, Liang Z, Zhuo W, Xie R, Zhu B (2013) Interleukin 17 produced by tumor microenvironment pro motes self renewal of CD133+ cancer stem like cells in ovarian can cer. Oncogene. doi:10.1038/onc.2013.537

65. Chen WC, Lai YH, Chen HY, Guo HR, Su IJ, Chen HH (2013) Interleukin 17 producing cell infiltration in the breast cancer tumour microenvironment is a poor prognostic factor. Histopathology 63(2):225 233

66. Chen Z, Ding J, Pang N, Du R, Meng W, Zhu Y, Zhang Y, Ma C, Ding Y (2013) The Th17/Treg balance and the expression of related cytokines in Uygur cervical cancer patients. Diagn Pathol 8:61. doi:10.1186/1746 1596 8 61

67. Ngiow SF, Teng MW, Smyth MJ (2013) Balance of interleukin 12 and 23 in cancer. Trends Immunol 34(11):548 555

Exhibit 72

Daniel L. Clarke-Pearson, M.D.

```
 1              IN THE UNITED STATES DISTRICT COURT

 2                 FOR THE DISTRICT OF NEW JERSEY

 3

      ----------------------------------X

 4

      IN RE:   JOHNSON & JOHNSON

 5

      TALCUM POWDER PRODUCTS

 6                                      MDL No.:

      MARKETING, SALES PRACTICES,

 7                                      16-2738 (FLW)(LHG)

      AND PRODUCTS LIABILITY

 8

      LITIGATION

 9

      ----------------------------------X

10

11           ORAL AND VIDEOTAPED DEPOSITION OF

12              DANIEL L. CLARKE-PEARSON, M.D.

13   _____

14                 MONDAY, FEBRUARY 4, 2019

15                      9:03 A.M.


      _____

16

                   Taken by the Defendants

17                   at The Carolina Inn

                     211 Pittsboro Street

18           Chapel Hill, North Carolina 27516

19

20                       -  -  -

21      Reported by Sophie Brock, RPR, RMR, RDR, CRR

22                       -  -  -

23

24             GOLKOW LITIGATION SERVICES

            877.370.3377 ph | 917.591.5672 fax

25                  deps@golkow.com
```

Daniel L. Clarke-Pearson, M.D.

Page 2

```
 1          A P P E A R A N C E S
 2  ON BEHALF OF THE PLAINTIFFS:
 3      BEASLEY, ALLEN, CROW, METHVIN,
        PORTIS & MILES, P.C.
 4      218 Commerce Street
        Montgomery, Alabama 36104
 5      Telephone: (334) 269-2343
        By:  LEIGH O'DELL, ESQ.
 6          leigh.odell@beasleyallen.com
            MARGARET THOMPSON, MD, JD, MPAff
 7          margaret.thompson@beasleyallen.com
 8      - and -
 9      BLOOD, HURST & O'REARDON, LLP
        501 West Broadway, Suite 1490
10      San Diego, California 92101
        Telephone: (619) 338-1100
11      By:  PAULA R. BROWN, ESQ.
            pbrown@bholaw.com
12
13  ON BEHALF OF THE DEFENDANT
    JOHNSON & JOHNSON:
14
        TUCKER ELLIS, LLP
15      515 South Flower Street
        Forty-Second Floor
16      Los Angeles, California 90071
        Telephone: (213) 430-3301
17      By:  MICHAEL C. ZELLERS, ESQ.
            michael.zellers@tuckerellis.com
18
        - and -
19
        DRINKER BIDDLE & REATH, LLP
20      600 Campus Drive
        Florham Park, New Jersey 07932-1047
21      Telephone: (973) 549-7164
        By:  JESSICA L. BRENNAN, ESQ.
22          jessica.brennan@dbr.com
23
24
25
```

Page 4

Page 3

```
 1      A P P E A R A N C E S  (Continued)
 2  ON BEHALF OF THE DEFENDANT
    IMERYS TALC AMERICA, INC.:
 3
        DYKEMA GOSSETT, PLLC
 4      112 E. Pecan Street, Suite 1800
        San Antonio, Texas 78205
 5      Telephone: (210) 554-5549
        By:  JANE E. BOCKUS, ESQ.
 6          jbockus@dykema.com
 7      - and -
 8      COUGHLIN DUFFY LLP
        350 Mount Kemble Avenue
 9      Morristown, New Jersey 07962
        Telephone: (973) 267-0058
10      By:  MARYAM M. MESEHA, ESQ.
            mmeseha@coughlinduffy.com
11
12  ON BEHALF OF THE DEFENDANT
    PERSONAL CARE PRODUCTS COUNCIL:
13
        SEYFARTH SHAW LLP
14      975 F Street, N.W.
        Washington, DC 20004-1454
15      Telephone: (202) 463-2400
        By:  JAMES R. BILLINGS-KANG, ESQ.
16          jbillingskang@seyfarth.com
17
18  ON BEHALF OF THE DEFENDANT PTI:
19      TUCKER ELLIS
        233 South Wacker Drive
20      Chicago, Illinois 60606
        Telephone: (312) 624-6300
21      By:  JAMES W. MIZGALA, ESQ.
            james.mizgala@tuckerellis.com
22
23  VIDEOGRAPHER:
24      Brad Smith
25
```

Page 5

Daniel L. Clarke-Pearson, M.D.

Page 6

Page 7

Page 8

1   P R O C E E D I N G S
2   THE VIDEOGRAPHER: We are now on
3   record. Today's date is February 4, 2019, and the
4   time is approximately 9:03 a m.
5   This is the videotaped deposition of
6   Dr. Daniel Clarke-Pearson. It's being taken in
7   regards to the Talcum Powder Litigation, MDL No. 2738.
8   Would counsel please now introduce
9   themselves for the record, and then our court reporter
10   will swear in the witness.
11   MS. O'DELL: Leigh O'Dell from
12   Beasley Allen, on behalf of the plaintiffs.
13   MS. THOMPSON: Margaret Thompson,
14   Beasley Allen, on behalf of the plaintiffs.
15   MS. BROWN: Paula Brown from Blood,
16   Hurst & O'Reardon, on behalf of the plaintiffs.
17   MR. ZELLERS: Michael Zellers, on
18   behalf of the Johnson & Johnson defendants.
19   MS. BRENNAN: Jessica Brennan, on
20   behalf of the Johnson & Johnson defendants.
21   MR. BILLINGS-KANG: James
22   Billings-Kang, Seyfarth Shaw, on behalf of Personal
23   Care Products Council.
24   MS. BOCKUS: Jane Bockus, on behalf of
25   Imerys.

Page 9

1   MS. MESEHA: Maryam Meseha, on behalf
2   of Imerys.
3   MR. MIZGALA: James Mizgala, on behalf
4   of PTI.
5   Whereupon,
6   DANIEL L. CLARKE-PEARSON, MD,
7   having first been duly sworn/affirmed,
8   was examined and testified as follows:
9   EXAMINATION BY COUNSEL FOR THE
10   JOHNSON & JOHNSON DEFENDANTS
11   BY MR. ZELLERS:
12   Q. Can you state your name, please.
13   A. Yes. Daniel Lyle Clarke-Pearson.
14   Q. Dr. Clarke-Pearson, we're here to take your
15   deposition in the talcum powder MDL litigation.
16   You're aware of that?
17   A. Yes, sir.
18   Q. You've given a number of depositions in the
19   past; is that right?
20   A. I have.
21   Q. You are familiar with the rules that we're
22   going to follow here today?
23   A. Yes.
24   Q. If I ask you a question or if any counsel
25   asks you a question that you don't understand, tell us

Daniel L. Clarke-Pearson, M.D.

Page 10

1 you don't understand and we'll repeat or rephrase the
2 question so it's clear to you.
3   Can you do that?
4   A. Yes, sir.
5   Q. If you answer a question, we're going to
6 assume that you understood it. Is that fair?
7   A. Fair.
8   MS. O'DELL: Objection.
9 BY MR. ZELLERS:
10   Q. As we go along, only one of us can speak at a
11 time. So please try to let me finish my question
12 before you answer. I will try to allow you to finish
13 your answer so that we can get the best record
14 possible.
15   Is that agreeable?
16   A. Agreeable.
17   Q. All right. You are following this,
18 apparently, on the realtime; is that right?
19   A. Yes.
20   Q. Is that going to be distracting to you?
21   A. It might be.
22   Q. All right. Well, have you ever done that
23 before in a deposition?
24   A. No, sir.
25   Q. Well, if it becomes distracting, then we'll

Page 11

1 deal with it.
2   You are here pursuant to a notice of
3 deposition. We've marked the notice of deposition as
4 Exhibit 1.
5   (Exhibit No. 1 was marked for identification.)
6 BY MR. ZELLERS:
7   Q. Can you take a look at that and let us know
8 if you've seen that before?
9   MS. O'DELL: I would just reassert that
10 the objections to certain document requests in the
11 notice, I think those were previously served.
12   MR. ZELLERS: Yes, we did receive the
13 objections of plaintiffs.
14   THE WITNESS: Yes, I've seen this.
15 BY MR. ZELLERS:
16   Q. If you go to -- beginning on page 3, there
17 are a number of documents that are requested be
18 produced here today.
19   Have you either brought with you here today
20 or supplied to counsel for plaintiffs all of the
21 documents and materials in your possession that are
22 requested in the deposition notice?
23   MS. O'DELL: To the degree that they
24 are not objectionable --
25   MR. ZELLERS: No. My question goes,

Page 12

1 you know, across the board. If there is a document
2 that he has in his possession that may be
3 objectionable, then he can tell us what it is and you
4 can assert your objection.
5   MS. O'DELL: Well, you asked if he had
6 brought them here, and Dr. Clarke-Pearson has only
7 brought materials subject to requests that are not
8 objectionable, which include the materials listed in
9 his materials-considered list that are in the binders
10 behind me on the table.
11   They also include binders of cited
12 materials, his report, invoices, and the cases in
13 which he has provided testimony within the last five
14 years. I think he has a copy of his report in front
15 of him.
16   Those are the materials we view to be
17 nonobjectionable, and those are what
18 Dr. Clarke-Pearson has brought with him today.
19   MR. ZELLERS: Okay. Ms. O'Dell, as
20 we -- I would appreciate it if you let the witness
21 answer the questions. I do appreciate the
22 clarification. But, as we go along today, if you'll
23 do your best, you know, to follow the rules. I mean,
24 the both of us need to follow in terms of objections.
25 I'd appreciate it.

Page 13

1   MS. O'DELL: Well, certainly, I'm going
2 to follow the rules today, but it's because of the
3 objections asserted and because it's unclear to what
4 degree Dr. Clarke-Pearson is familiar with all the
5 requests and all the objections, then that was just a
6 difficult question for him -- maybe an unfair question
7 for him. And so I have responded in keeping with our
8 previously served objections.
9   MR. ZELLERS: I don't think asking him
10 if he's gone through the request for production of
11 documents and can identify for us any documents that
12 are in your possession that are responsive that you've
13 not brought here today, I don't think that is a
14 difficult question. But let's have Dr. Clarke-Pearson
15 answer it.
16   THE WITNESS: I don't think I've
17 brought any of these documents here today. Counsel
18 has some of them, like my curriculum vitae.
19 BY MR. ZELLERS:
20   Q. My question, I guess, goes to -- so that we
21 can identify whether there's anything at all for us
22 that we need to fight about should be produced.
23   Are there documents that are responsive to
24 the notice of deposition that are not being produced
25 here today, to your knowledge, that originated from

Daniel L. Clarke-Pearson, M.D.

Page 14

1 you and are in your possession?
2   A. I think let's just walk through the list,
3 then. I don't have a CV in my possession, but counsel
4 does --
5   Q. And, Doctor, to shortcut this, I don't need
6 to go through and ask you, you know, what documents
7 are being produced.
8   Are you aware of documents that are called
9 for in the notice of deposition that are not being
10 produced today?
11   A. I don't -- I would have to go through this
12 list. I don't have any documents with me aside from
13 what you've just described.
14   Q. So you've reviewed the notice of deposition
15 in preparation for today; correct?
16   A. Yes.
17   Q. You knew that was important; correct?
18   A. Yes.
19   Q. And yet you're unable to tell us whether or
20 not there are documents that are in your possession
21 that are called for in the notice of deposition that
22 you are not producing today; is that right?
23   MS. O'DELL: Objection. That's not
24 correct, but --
25   MR. ZELLERS: Well, he can answer.

Page 15

1   MS. O'DELL: I've made my objection --
2   MR. ZELLERS: Understood.
3   MS. O'DELL: -- which I'm perfectly
4 entitled to do that, as you know.
5   MR. ZELLERS: You certainly are. You
6 certainly are.
7   MS. O'DELL: So, Dr. Clarke-Pearson,
8 just answer to the best of your knowledge, and, of
9 course, there are objections that have been asserted;
10 and to the degree you're not familiar with those
11 details, then counsel and I can sort that out later.
12   THE WITNESS: So documents -- I do not
13 have any of these documents in my possession. For
14 example, I thought I saw -- passed you a document
15 showing my billing and collections to date. Isn't
16 that right on top?
17 BY MR. ZELLERS:
18   Q. My question was are you aware, as you sit
19 here right now, of any documents that you have that
20 are responsive to the notice of deposition that are
21 not in the large pile of materials that we have here
22 today?
23   A. I'm not aware of any.
24   Q. All right. Ms. O'Dell produced for us or
25 provided to me two documents prior to the deposition

Page 16

1 and then has advised me that you have reviewed a
2 number of additional materials since you prepared your
3 report. So I'd like to go through those now, if we
4 can.
5   Notice of deposition, Exhibit 2, is a copy,
6 it appears, of your invoices in this matter. Is that
7 right?
8   (Exhibit No. 2 was marked for identification.)
9   THE WITNESS: Yes, sir.
10 BY MR. ZELLERS:
11   Q. You have spent a total of 20 hours working on
12 this matter since being retained back in April of
13 2017; is that right?
14   MS. O'DELL: Object to the form.
15   THE WITNESS: Up until the preparation
16 of -- and submission of my report, I spent 20 hours.
17 BY MR. ZELLERS:
18   Q. All right. You prepared your report, you
19 edited your report, and you submitted your report on
20 November 4th of 2018; is that right?
21   A. I believe it was -- I submitted it, but
22 I think it was November 16th, 2018.
23   Q. Did you bill any time or spend any time on
24 the MDL talcum powder litigation between
25 November 4th of 2018 and the end of the year,

Page 17

1 December 31st of 2018?
2   A. Yes.
3   Q. How much additional time did you spend during
4 that time?
5   A. I don't know exactly. I'd have to go back to
6 several notes that I have on records and papers and
7 that sort of thing. I would say between
8 November 4th and today, it's been about 60 hours.
9   Q. 60 additional hours?
10   A. Yes, sir.
11   Q. So you spent 20 hours talking with counsel,
12 doing whatever research and analysis you needed to do,
13 and writing your report; is that right?
14   A. Yes.
15   Q. You have spent an additional 60 hours since
16 that time; is that right?
17   A. Yes.
18   Q. If your invoice is dated January 4th of 2019,
19 Exhibit 2, why does none of that time appear on your
20 invoice?
21   A. Because my accounting office turned this over
22 on January 4th. I submitted -- I submitted this
23 invoice to my business manager, and this is when it
24 was submitted from our office.
25   Q. I guess I don't understand. You tell me that

Daniel L. Clarke-Pearson, M.D.

Page 18	Page 20
1 you have worked a considerable amount of time between	1 Ms. O'Dell -- strike that -- with Dr. Thompson over
2 November 4th of 2018 and the end of 2018; correct?	2 the years?
3 A. Yes.	3 A. I believe she probably called me somewhere
4 Q. Why is that time and those hours not	4 before April 17th when I was retained and described
5 reflected on your invoice which is dated January 4th	5 work that was ongoing with talcum powder. So we had a
6 of 2019?	6 conversation. I didn't bill for that.
7 A. Because I hadn't submitted the request for my	7 Q. You knew Dr. Thompson socially before being
8 business manager to submit the invoice to the	8 retained; is this correct?
9 attorneys.	9 A. Yes.
10 Q. Why did you cut off your time entry as of	10 Q. Other than --
11 November 4th, 2018?	11 A. And -- excuse me. And professionally.
12 MS. O'DELL: Object to the form.	12 Q. Socially and professionally.
13 THE WITNESS: I think there was a gap.	13 What professional interaction did you have
14 I can't tell you when I picked up again after	14 with Dr. Thompson since the time that you were a
15 November 4th, after I did the report. There was a	15 resident and a fellow at Duke University?
16 time when I wasn't actively involved reading,	16 A. Okay. So since that time -- I mean,
17 preparing.	17 throughout her residency, we were professionally
18 BY MR. ZELLERS:	18 involved with training and taking care of patients.
19 Q. Do you keep track of the time that you spend	19 Subsequent to her completing her residency, I've not
20 doing activities as an expert witness in the MDL	20 had any professional interaction with her per se.
21 talcum powder litigation?	21 Q. Were you socially involved with Dr. Thompson
22 A. Yes.	22 while the two of you were at Duke?
23 Q. And do you keep that on a regular, systematic	23 A. No.
24 basis?	24 Q. You might go to events and see one another,
25 A. Not so much.	25 but in terms of any relationship between the two of

Page 19	Page 21
1 Q. Were you first retained back in April of 2017	1 you, there was none; is that fair?
2 by Ms. O'Dell and by Ms. Thompson?	2 A. I guess you'll have to define "relationship"
3 A. Yes, I believe so.	3 for me.
4 Q. Had you known Ms. O'Dell or any attorneys	4 Q. Well, I was trying to make it easy.
5 from her office, the Beasley Allen office, prior to	5 Did you socialize with other persons in the
6 being contacted in this litigation?	6 internship and residency programs while you were at
7 A. I had not known Ms. O'Dell. I knew	7 Duke?
8 Dr. Thompson.	8 A. Yes. And faculty and spouses, yes.
9 Q. How did you know Dr. Thompson?	9 Q. And Dr. Thompson was one of those persons; is
10 A. Dr. Thompson and I were residents at Duke	10 that right?
11 University Medical Center. I was a few years ahead of	11 A. Yes, sir.
12 her, but we were in the residency training program.	12 Q. Do you know Dr. Thompson's husband or former
13 And then I began my fellowship and gynecologic	13 husband?
14 oncology at Duke, and I believe Dr. Thompson was still	14 A. I did not.
15 a resident during part of that time.	15 Q. All right. Your contact was solely with
16 Q. Did you make -- maintain contact with	16 Dr. Thompson; is that right?
17 Dr. Thompson over the years?	17 A. Yes.
18 A. Off and on. Probably on average about once a	18 Q. Over the years, prior to being retained by
19 year at an alumni meeting that we attended, although	19 Dr. Thompson in this litigation, did you review any
20 neither one of us attended every year, but...	20 medicolegal matters for her?
21 Q. These were alumni meetings at Duke	21 A. No, sir.
22 University; is that right?	22 Q. Were you asked to review any medicolegal
23 A. With regard to the obstetrical and	23 matters for her?
24 gynecological department.	24 A. You just asked that question, I think.
25 Q. Other contacts that you had with	25 Q. No --

Daniel L. Clarke-Pearson, M.D.

Page 22

1   A. Did I misunderstand?
2   Q. Well, and at least what I had hoped was the
3 distinction is that I had asked you if you had
4 reviewed any matters, and then the second question was
5 whether or not Dr. Thompson had requested that you
6 review any medicolegal matters for her.
7   A. Okay. So it's a two-part question. I did
8 not review any matters, and Dr. Thompson hadn't
9 requested me to review any medicolegal matters.
10   Q. When -- well, strike that.
11   What did Dr. Thompson ask you to do with
12 respect to the MDL talcum powder litigation?
13   A. At the time of the conference call with
14 Ms. O'Dell and Dr. Thompson, I was asked to evaluate
15 and offer my opinion regarding talcum powder and
16 whether it was causative to the occurrence of ovarian
17 cancer in women who use talcum powder on their
18 perineum.
19   Q. Were you asked to research or answer any
20 other question other than that?
21   A. So in my report, I think I make it clearer
22 than what I just described. So "Can the use of talcum
23 powder in the perineal area cause epithelial ovarian
24 cancer?" and also, "If so, what biologic mechanism did
25 this -- by which did this occur?" were the two key

Page 23

1 questions I was asked to form an opinion on.
2   Q. You mentioned that you did speak with
3 Dr. Thompson prior to the conversation with Ms. O'Dell
4 and Dr. Thompson.
5   What, at that time, did Dr. Thompson tell
6 you about the litigation?
7   A. I don't recall details. It was that she was
8 working on cases that had to do with talcum powder and
9 ovarian cancer.
10   Q. Do you recall any other background that you
11 were provided?
12   A. Not at that time.
13   Q. Did you understand that Dr. Thompson was
14 representing the plaintiffs in this matter, along with
15 a number of other attorneys?
16   A. Yes.
17   Q. Prior to being contacted by Dr. Thompson and
18 by Ms. O'Dell, had you formed opinions in terms of
19 whether or not talcum powder was causally related to
20 ovarian cancer for women who used it in the perineal
21 region?
22   A. So that's an interesting question, because it
23 goes back to my training. And throughout the years,
24 since 1975, when I began my residency training, the
25 conversation in the gynecologic community and the

Page 24

1 GYN oncology community has been one of could talcum
2 powder be associated with the occurrence of ovarian
3 cancer?
4   And, in fact, I think, in the early '70s, we
5 believed it did; and then I was told as a trainee that
6 talcum powder previously had had asbestos in it, and
7 then we were told it was taken out. So that was very
8 reassuring.
9   Yet periodically over the years, papers came
10 out -- case-control studies, cohort studies -- off and
11 on that continued to raise the question.
12   So the question has been in my mind. And,
13 really, it wasn't until I really started thinking
14 about this and gathered up all the literature that it
15 became clear to me, and I formed my opinion.
16   Q. That was my question. When did you form your
17 opinion that talcum powder is causally related to
18 ovarian cancer when used by women in the genital area?
19   A. Well, some -- I'm not sure there was a
20 particular day when the light bulb went off. I think
21 in the process of digging into this issue in more
22 detail and putting together all the case-control
23 trials that had come out over a period of time and the
24 meta-analysis that had come out over a period of time
25 that kept raising questions, when I started to put

Page 25

1 that all together, it became clear to me that, in my
2 opinion, talcum powder causes ovarian cancer.
3   Q. That was sometime after you were contacted
4 and retained in this matter back in April of 2017 as
5 an expert for the plaintiffs; correct?
6   A. It was the request to provide opinions and to
7 develop an opinion, and I -- yes.
8   Q. All right. Do you agree that the medical
9 community as a whole has not reached a consensus that
10 talcum powder causes ovarian cancer?
11   MS. O'DELL: Object to the form.
12 Excuse me.
13   THE WITNESS: I think we're at a
14 tipping point in that question.
15 BY MR. ZELLERS:
16   Q. Can you answer that question?
17   A. Well, I think you would have to define "the
18 medical community" for me.
19   Q. Well, let's be more specific.
20   Has the gynecologic oncologist medical
21 community reached a consensus that talcum powder
22 causes ovarian cancer?
23   A. As best I know, not at this time.
24   Q. All right. You also -- Ms. O'Dell provided
25 me with an updated list of your testimony; is that

Daniel L. Clarke-Pearson, M.D.

Page 26

1 right?
2   MR. ZELLERS: We'll mark that as
3 Exhibit 3.
4   (Exhibit No. 3 was marked for identification.)
5   THE WITNESS: Yes, sir.
6 BY MR. ZELLERS:
7   Q. The testimony that you provided back in
8 November of 2017 -- strike that -- November of 2018,
9 when you submitted your report, Exhibit C -- we'll
10 mark that as Deposition Exhibit 4 --
11   (Exhibit No. 4 was marked for identification.)
12   Q. -- contained just one listing of testimony;
13 is that right?
14   A. Yes.
15   Q. What has changed since you prepared your
16 report in November of 2018 and today with respect to
17 deposition and trial testimony that you have provided?
18   A. I believe simply an oversight on my part.
19   Q. The oversight was not listing at least two of
20 the matters that you had testified in in the past five
21 years as of November of 2018; is that right?
22   A. Yes, sir.
23   Q. The Edmonson matter that you testified in
24 December of 2014, was that a medical malpractice
25 action?

Page 27

1   A. Yes, it was a malpractice action.
2   Q. And September 1st of 2015, the Rappaport
3 matter, that was a physician who was losing his or her
4 privileges?
5   A. He was being fired from his practice.
6   Q. The Pizzirusso case or matter that you
7 provided testimony in March of 2015, what was that?
8   A. That was a medical malpractice case in
9 Brooklyn, New York.
10   Q. January of 2019, Paduda, what type of matter
11 was that?
12   A. This was -- I need to make sure I've got the
13 two straight here. Yes, it's a medical malpractice
14 case.
15   Q. And then, finally, you were deposed on
16 January 22nd of 2009 in a matter called Cutsinger.
17 What type of matter was that?
18   A. It was 2019.
19   MS. O'DELL: '19.
20   MR. ZELLERS: Thank you, Counsel.
21 BY MR. ZELLERS:
22   Q. January 22nd of 2019.
23   A. This is a product liability suit.
24   Q. Involving what product?
25   A. A morcellator manufactured by Gyrus.

Page 28

1 BY MR. ZELLERS:
2   Q. The medical malpractice cases that you have
3 listed -- Edmonson, Pizzirusso, and Paduda -- were you
4 serving as an expert for plaintiff or defense in those
5 cases?
6   A. In all three of those cases, for the defense.
7   Q. Over the years, you have done a lot of
8 testifying in medical malpractice cases. Is that
9 fair?
10   MS. O'DELL: Object to the form.
11   THE WITNESS: I don't know how you
12 define "a lot."
13 BY MR. ZELLERS:
14   Q. Have you given -- at least up until 2005 or
15 so, did you give about three depositions a year in
16 medical malpractice cases?
17   A. Probably three or more. Three to six, maybe.
18   Q. Since 2005, you've cut back some in terms of
19 your medicolegal work; is that right?
20   A. Yes.
21   Q. Is it accurate to say that, over the years,
22 you've testified about 50 percent for plaintiff and
23 about 50 percent for defendants in litigation matters?
24   A. Yes.
25   Q. Is the only product liability matter that you

Page 29

1 have testified in, other than the MDL talcum powder
2 litigation, the morcellator deposition that you gave
3 earlier in -- this year, in January?
4   A. Yes, sir.
5   Q. Ms. O'Dell advised us at the start of the
6 deposition that, in addition to the materials that you
7 cite in your report and in your additional materials
8 list, that you have now reviewed a number of
9 additional materials prior to today; is that right?
10   A. Yes.
11   Q. Do those additional materials that you have
12 reviewed change in any respect the opinions that you
13 have set forth in your report?
14   A. They reinforce and enhance or support my
15 opinion.
16   Q. As we go through today, I may refer to talc,
17 I may refer to talcum powder, I may refer to talc
18 products or to baby powder or to Shower to Shower.
19 I intend, when I use those terms, to be referring to
20 the baby powder product manufactured by Johnson &
21 Johnson Consumer Products Inc. and the Shower to
22 Shower product formerly manufactured by Johnson &
23 Johnson Consumer Products Inc.
24   Do you understand that?
25   A. I understand.

Daniel L. Clarke-Pearson, M.D.

Page 30

1   Q. Your report which was provided to us, we will
2 mark as Deposition Exhibit 5.
3   (Exhibit No. 5 was marked for identification.)
4 BY MR. ZELLERS:
5   Q. Can you just take a quick look at that and
6 confirm for us that that is Deposition Exhibit 5?
7   A. It is.
8   Q. Your report, which we have marked as
9 Deposition Exhibit 5, does that contain all of the
10 opinions that you intend to offer at any trial or
11 hearing in this matter?
12   A. I believe so, yes.
13   Q. Does your report identify everything that you
14 are relying on in forming your opinions in this
15 matter?
16   MS. O'DELL: Object to the form.
17   THE WITNESS: Obviously, we just talked
18 about some additional information -- materials that
19 I've reviewed since writing that report, so they would
20 be included in my opinion.
21 BY MR. ZELLERS:
22   Q. We'll go through in a moment the additional
23 materials that you have reviewed.
24   If we look at your report and if we look at
25 the additional materials that you have reviewed in

Page 31

1 preparation for the deposition, does that include all
2 of the materials that you are relying on in forming
3 your opinion?
4   A. To be clear, you're saying what I have
5 included in my report plus my additional materials,
6 that's what I relied on?
7   Q. Yes.
8   Is that correct?
9   A. Yes.
10   Q. Is your report accurate?
11   A. Yes.
12   Q. Is your report complete?
13   A. I believe it is.
14   Q. Let's try to quickly go through, if we can,
15 the additional materials that you have reviewed since
16 you prepared your report, Exhibit 5.
17   Ms. O'Dell stated that you have reviewed the
18 Health Canada risk assessment; is that right?
19   A. Yes.
20   Q. You have reviewed the Taher, T-A-H-E-R, 2018
21 publication; is that right?
22   A. Yes.
23   Q. You have reviewed the 2019 Saed paper?
24   A. Yes.
25   Q. You have reviewed the Longo supplemental

Page 32

1 report?
2   A. Yes.
3   Q. You've reviewed a chapter of a book by
4 Creasman that was authored by Dr. Brewster; is that
5 right?
6   A. That's correct.
7   Q. Is there anything else that you have reviewed
8 and are relying on in preparation for your deposition
9 today and in providing us with your opinions?
10   A. So all these references here (indicating),
11 I've reviewed. I believe they're listed as part of an
12 exhibit.
13   Q. And let's, you know, be as systematic as we
14 can be.
15   Your report, Exhibit 5, has a list of
16 references; is that right?
17   A. Yes.
18   Q. What do you intend -- or what is the meaning
19 of the references that appear as pages 11 through 14
20 in your report?
21   A. Those references support what I quote -- not
22 quotes, but facts that are in my report. They don't
23 include everything that I used in my consideration of
24 coming to my opinion.
25   Q. Deposition Exhibit 6 is Exhibit B to your

Page 33

1 report.
2   (Exhibit No. 6 was marked for identification.)
3 BY MR. ZELLERS:
4   Q. Is that correct?
5   Is Deposition Exhibit B a listing of
6 additional materials considered?
7   A. Yes, it is.
8   Q. Did you actually read and consider all of the
9 materials that are cited as Exhibit B to your report?
10   A. I would say I did not read every word of
11 every paper. I reviewed them, many times reading the
12 abstract.
13   Q. Did you read at least the abstract of each of
14 the references contained as Exhibit B to your report,
15 going from page 1 through page 28?
16   A. I believe so.
17   Q. Exhibit B is meant to be materials that you
18 considered but are not directly relying on in
19 formulating your opinions; is that fair?
20   MS. O'DELL: Object to the form.
21   THE WITNESS: That's fair.
22 BY MR. ZELLERS:
23   Q. In addition to the references that are
24 attached to your report, Exhibit 5 to the deposition,
25 and Exhibit B, which we've marked as Exhibit 6 to the

Daniel L. Clarke-Pearson, M.D.

Page 34

1 deposition, are there any other materials that you
2 have reviewed and relied upon in formulating the
3 opinions you're going to give today other than the
4 additional materials that we discussed a moment ago?
5   A. No.
6   Q. Are there any additional materials that you
7 have reviewed and relied upon since the time of your
8 report other than the materials that have been
9 identified by Ms. O'Dell?
10   A. No.
11   Q. Did you bring those additional materials with
12 you in the folders that you have in front of you?
13   A. Some of them. I have the Longo updated
14 report, for example.
15   Q. All right. I'd like to just mark, so that we
16 have a record of what it is you have reviewed, to the
17 extent there's any ambiguity in the record. And, for
18 example, I'm looking at --
19   MS. O'DELL: Mike, excuse me. Can
20 I just mention one thing?
21   MR. ZELLERS: Yes.
22   MS. O'DELL: Because when you were
23 going through your list, I had mentioned before an
24 UpToDate reference. It's in the stack I think you
25 have in your hand. But you didn't mention that in

Page 35

1 your sort of questions to Dr. Clarke-Pearson. So
2 I don't want there to be a misrepresentation --
3   MR. ZELLERS: Understood.
4   MS. O'DELL: -- on the -- I didn't mean
5 it that way. I didn't want there to be a
6 misunderstanding on the record.
7   MR. ZELLERS: I do understand.
8 I appreciate the clarification.
9 BY MR. ZELLERS:
10   Q. What I had been given was a clip with the
11 Brewster chapter from the Creasman textbook. But in
12 addition to what was on top, there is an UpToDate
13 official reprint that states at the top
14 "Evidence-based medicine," and then it lists several
15 authors, the first of which is Arthur T. Evans; is
16 that correct?
17   A. Yes.
18   Q. That's an additional set of materials that
19 you have reviewed and relied upon?
20   A. Yes.
21   Q. Also in the stack, and something that I did
22 not mention earlier, is "Emerging Themes in
23 Epidemiology, Analytical Perspective." First author
24 is Fedak. And this appears to be a 2015 publication.
25   Is that also something that you reviewed and

Page 36

1 you relied upon?
2   A. Yes, sir.
3   Q. We'll mark the Brewster chapter as Exhibit 7.
4   (Exhibit No. 7 was marked for identification.)
5   MR. ZELLERS: We will mark the UpToDate
6 reprint as Exhibit 8.
7   (Exhibit No. 8 was marked for identification.)
8   MR. ZELLERS: We will mark the Emerging
9 Themes in Epidemiology, 2015, Fedak, as Exhibit 9.
10   (Exhibit No. 9 was marked for identification.)
11 BY MR. ZELLERS:
12   Q. I'll return these to you, Doctor.
13   Can you show me or provide to me whatever
14 folders you have brought. I don't need the binders,
15 but just whatever additional materials you have
16 brought with you.
17   (Document was handed to counsel.)
18 BY MR. ZELLERS:
19   Q. And then it looks like you have IARC
20 monographs; is that right?
21   A. Yes.
22   Q. Are those IARC monographs that you have
23 brought with you, is that something that's either on
24 your reference list or your reliance list?
25   A. I believe it is.

Page 37

1   Q. Can you just tell us the title of the IARC
2 monograph that you have brought with you?
3   A. "IARC Monographs on the Evaluation of
4 Carcinogenic Risks to Humans, Volume 93, Carbon Black,
5 Titanium Dioxide, and Talc," dated 2010.
6   Q. The next set of materials, I'll mark these
7 collectively as Exhibit 10 so we can keep them in the
8 same order that you have brought them with you.
9   (Exhibit No. 10 was marked for identification.)
10 BY MR. ZELLERS:
11   Q. Exhibit 10, the first page is a listing of
12 handwritten notes. Can you read just the first line
13 to us.
14   A. "Exposure IARC 100C page 232."
15   Q. What does that refer to?
16   A. I put these together, if I can explain, so
17 that we might facilitate this discussion and be able
18 to find documents a little bit more quickly.
19   Q. What discussion does Exhibit 10 relate to?
20   A. Could I see the front of the folder, please?
21   Q. Sure.
22   A. It has to do with asbestos and ovarian
23 cancer.
24   Q. I will re-mark Deposition Exhibit 10.
25 Instead of putting the sticker on your page of

Daniel L. Clarke-Pearson, M.D.

Page 38

1 handwritten notes, I'll put it on the outside of the
2 folder, which are your references on asbestos and
3 ovarian cancer; is that right?
4   MS. O'DELL: Object to the form.
5   THE WITNESS: They are some of my
6 references.
7 BY MR. ZELLERS:
8   Q. These are the references, though, that you
9 chose to bring with you today to be prepared to answer
10 questions that the lawyers may ask?
11   MS. O'DELL: Object to the form. He
12 brought other references as well.
13   THE WITNESS: All of these references
14 here are -- also could support the question in that
15 folder about asbestos and ovarian cancer.
16 BY MR. ZELLERS:
17   Q. Who prepared the folder "Asbestos on Ovarian
18 Cancer"?
19   A. I did.
20   Q. Whose notes are the first page of this
21 folder?
22   A. That's mine.
23   Q. Who chose to include and to write down the
24 references that you did on this piece of paper?
25   A. I did.

Page 39

1   Q. The other exhibits that you have today, the
2 exhibits that we marked, was it -- Exhibit 9, is that
3 the Brewster chapter?
4   A. Exhibit 7 is the Brewster chapter.
5   Q. Okay, Exhibit 7. Who provided those
6 materials to you?
7   A. This is from a textbook in my office.
8   Q. Okay. Did you obtain that -- you know, that
9 information?
10   A. I'm not quite sure -- so I wrote a chapter
11 for this textbook myself on surgical complications.
12 It's a textbook that's in my office. This particular
13 document, if you will, or reprint from that chapter,
14 I'm not sure if I produced it or counsel did.
15   Q. Well, it's clear at the bottom that it was
16 produced by counsel; correct?
17   A. Okay.
18   Q. There's a notation that Dr. Thompson
19 downloaded that reference back in January of this
20 year; is that right?
21   A. I see that, yes.
22   Q. Are many of the materials that you've looked
23 at, including those on your reference list, your
24 additional materials-considered list, materials that
25 were provided to you by counsel for the plaintiffs?

Page 40

1   MS. O'DELL: Object to the form.
2   THE WITNESS: Many of them were
3 reprints that I created, and some were given to me by
4 counsel.
5 BY MR. ZELLERS:
6   Q. Are you able -- if we went through your list
7 of references that are attached to your report,
8 Exhibit 5, are you able to tell me easily which ones
9 came from counsel and which ones you may have found on
10 your own?
11   A. No, not easily.
12   Q. All right. Same question with respect to
13 Exhibit B, this 28 pages of additional materials. Are
14 you able to separate out for us easily what materials
15 came from counsel and what materials you found on your
16 own?
17   MS. O'DELL: Object to the form.
18   THE WITNESS: No, I can't.
19 BY MR. ZELLERS:
20   Q. The materials that are included in Deposition
21 Exhibit 10, the articles that you list on your sheet
22 of paper and have brought with you, there is a -- it
23 looks like an excerpt from the IARC working group
24 relating to asbestos and different types of asbestos;
25 is that right?

Page 41

1   A. Yes.
2   Q. You're not an expert in asbestos; correct?
3   MS. O'DELL: Object to the form.
4   THE WITNESS: It seems like I've become
5 pretty good at it after reading all of this material.
6 BY MR. ZELLERS:
7   Q. Well, I understand that. But you do not hold
8 yourself out or consider yourself to be an expert in
9 asbestos; is that right?
10   A. I think I've made it part of my job as an
11 expert to become very familiar with the issues
12 regarding asbestos and ovarian cancer.
13   Q. Do you consider yourself to be an expert in
14 asbestos?
15   A. Can you define "expert," please.
16   Q. Sure. Are you an expert in the different
17 types of asbestos: chrysotile, amosite,
18 crocidolite, tremolite, actinolite, and anthophyllite?
19   A. I'm aware that there are different types of
20 asbestos.
21   Q. Are you an expert in it?
22   MS. O'DELL: Object to the form.
23   THE WITNESS: I'm not sure I understand
24 what an expert is.
25

Daniel L. Clarke-Pearson, M.D.

Page 42

BY MR. ZELLERS:

1 BY MR. ZELLERS:
2   Q. You're testifying as an expert gynecologist
3 oncologist in this case; is that right?
4   A. Yes.
5   Q. You consider yourself to be an expert in that
6 field; is that right?
7   A. Of course.
8   Q. Do you consider yourself to be an expert, to
9 provide expert testimony to the jury, on asbestos and
10 the different forms of asbestos?
11   A. I think I can testify to the jury what is in
12 the literature and the impact that asbestos has on
13 ovarian cancer risk.
14   Q. Prior to being retained by Dr. Thompson and
15 Ms. O'Dell, did you have professional experience with
16 asbestos?
17   A. I'm not sure what you mean by "professional
18 experience." I don't use it in my practice.
19   Q. Did you research it?
20   A. As I said, back in 1975, when I was a
21 resident, there was discussion about asbestos in
22 talcum powder.
23   Q. Did you consider yourself to be an expert in
24 asbestos before you were retained by Dr. Thompson and
25 Ms. O'Dell?

Page 43

1   MS. O'DELL: Object to the form.
2   THE WITNESS: I was aware of issues
3 with asbestos in terms of carcinogenic potential for
4 mesothelioma and ovarian cancer.
5 BY MR. ZELLERS:
6   Q. Is that a yes, you considered yourself to be
7 an expert in asbestos prior to being retained in this
8 matter?
9   MS. O'DELL: Object to the form.
10 I think he stated he was an expert in the health
11 effects.
12   MR. ZELLERS: The doctor can answer the
13 questions.
14   MS. O'DELL: He did answer the
15 question.
16   THE WITNESS: That's what I was trying
17 to say. It was the health effects, carcinogenic
18 potential of asbestos in talcum powder and other
19 industrial exposures.
20 BY MR. ZELLERS:
21   Q. Are you familiar with at least what the
22 different types of claimed asbestos is in talcum
23 powder?
24   A. Yes.
25   Q. And are you familiar with the health effects

Page 44

1 or alleged health effects of those different types of
2 asbestos?
3   A. Yes.
4   Q. Did you consider yourself to be an expert in
5 asbestos prior to being retained in this litigation in
6 2017?
7   MS. O'DELL: Objection. Asked and
8 answered.
9   THE WITNESS: I don't know when
10 I morphed into feeling I knew more about asbestos than
11 I did in 1975.
12 BY MR. ZELLERS:
13   Q. Your -- the -- strike that.
14   What gives you expertise, in your view, as
15 an expert in asbestos is the reading that you have
16 done since being retained in this matter; is that
17 right?
18   MS. O'DELL: Objection to the form.
19 Misstates his testimony.
20   THE WITNESS: The knowledge that I've
21 gained over time, including during this preparation
22 for this deposition and my report.
23 BY MR. ZELLERS:
24   Q. When you were contacted by Dr. Thompson, did
25 you consider yourself to be an expert in asbestos at

Page 45

1 that time?
2   MS. O'DELL: Object to the form.
3   THE WITNESS: Again, I've told you what
4 I knew about asbestos at that time, and I've learned
5 more since then.
6 BY MR. ZELLERS:
7   Q. Can you answer my question?
8   Did you consider yourself to be an expert in
9 asbestos when you were first contacted by
10 Dr. Thompson?
11   A. Again, I'm stuck with what -- how you define
12 asbestos -- how you define an expert.
13   Q. You're an expert who -- an expert is someone
14 who has a special expertise in a matter that peers
15 would look to as a person and a resource.
16   Do people look to you as a resource on
17 asbestos?
18   A. People looked to me for a long time with
19 regard to -- as a resource with regard to asbestos and
20 its effects on the female genital tract and ovarian
21 cancer.
22   Q. So that's a yes, people have come to you for
23 some number of years as an expert on asbestos?
24   A. Patients have.
25   MS. O'DELL: Object to the form. It

Daniel L. Clarke-Pearson, M.D.

Page 46

1 misstates his testimony.
2   MR. ZELLERS: Well, I'm trying to get
3 an answer to my question.
4   MS. O'DELL: I think he answered your
5 question.
6   THE WITNESS: Patients have come to me
7 as an expert in this topic as it relates to their
8 health.
9 BY MR. ZELLERS:
10   Q. How about your peers? Do your peers come to
11 you as an expert in asbestos at any time?
12   A. I have different groups of peers. My
13 gynecologic oncology colleagues, I don't think I'm any
14 more of an expert than they are.
15   On the other hand, a general obstetrician
16 and gynecologist, an internist, a family medicine
17 physician, a pediatrician would consider me an expert.
18   Q. And that -- so my question very simply is do
19 your peers come to you as an expert in asbestos?
20   MS. O'DELL: Object to the form. Asked
21 and answered.
22   THE WITNESS: I have lots of different
23 levels of peers, is what I was trying to describe.
24 BY MR. ZELLERS:
25   Q. The second article that you brought and

Page 47

1 placed in your "Asbestos Ovarian Cancer" folder is an
2 article by Reid. States at the top, published online
3 first May 24, 2011, in Cancer Epidemiology,
4 "Biomarkers & Prevention"; is that right?
5   A. Yes.
6   Q. The third article is "Occupational Exposure
7 to Asbestos and Ovarian Cancer." This is a paper with
8 the first author of Camargo. It appears that it was
9 published in Environmental Health Perspectives,
10 September 2011; is that right?
11   A. Yes.
12   Q. The last paper that you included in your
13 folder was an article on ovarian cancer and asbestos,
14 first named author Graham. It was received -- is this
15 1967?
16   A. Yes, sir.
17   Q. You brought with you, which we will mark as
18 Exhibit 11, a folder captioned "EPI." Is that right?
19   A. Yes.
20   (Exhibit No. 11 was marked for identification.)
21 BY MR. ZELLERS:
22   Q. The first page, are these your notes to help
23 you in terms of answering my questions relating to the
24 epidemiology of ovarian cancer and talcum powder?
25   A. Yes, sir.

Page 48

1   Q. Did you prepare these notes?
2   A. Yes.
3   Q. First paper you list here is -- or have
4 brought with you included in this folder and
5 highlighted is Gates, which was published
6 November 12th of 2009; is that right?
7   A. Yes.
8   Q. You also have brought a paper, HHS Public
9 Access, "Douching, Talc Use," Epidemiology, 2016.
10 First author is Gonzalez; is that right?
11   A. Yes, sir.
12   Q. Then you have another collection of materials
13 with some additional handwritten notes, also in what
14 we have marked as Exhibit 11, your "EPI" folder. And
15 at the top of your handwritten notes, which appear on
16 two Post-its, it's "Penninkilampi."
17   That is a study that you have written down
18 along with some other notes, and you have brought that
19 with you in your folder; is that right?
20   A. Yes.
21   Q. You have brought the Berge paper, dated
22 May 18, 2018, European Journal of Cancer Prevention.
23 You have that in your folder; correct?
24   A. Yes.
25   Q. You have the Langseth paper that was accepted

Page 49

1 for -- well, strike that -- that was published in
2 Journal of Epidemiol. Community Health, 2008; is that
3 right?
4   A. Yes.
5   Q. And then finally, you have in your folder the
6 Taher -- T-A-H-E-R -- paper, which appears to be -- is
7 this a 2018 or 2019 paper, if you know?
8   A. I don't know.
9   Q. Was the Taher paper something that was
10 provided to you by counsel for the plaintiffs?
11   A. Yes.
12   Q. Was the Health Canada assessment something
13 that was provided to you by counsel for plaintiffs?
14   A. Yes.
15   Q. You've got a folder on animals with a couple
16 of very brief notes. We've marked your folder on
17 animals as Exhibit 12.
18   (Exhibit No. 12 was marked for identification.)
19 BY MR. ZELLERS:
20   Q. First paper we have is the Keskin article
21 from Gynecologic Obstetrics, 2009. Keskin is spelled
22 K-E-S-K-I-N. Is that right?
23   A. Yes, the spelling's correct.
24   Q. The next paper is the Hamilton paper. It
25 looks like it was published in 1984. The other

Daniel L. Clarke-Pearson, M.D.

Page 50

1 authors are Fox, Buckley, Henderson, and Griffiths.
2 It was received for publication in 1983.
3   Is that right?
4   A. Yes.
5   Q. Are these studies that you found, these
6 animal studies, or are these studies that were
7 provided to you by counsel for the plaintiffs?
8   MS. O'DELL: Object to the form.
9   THE WITNESS: I think it's some of
10 both.
11 BY MR. ZELLERS:
12   Q. Well, there's only two that are here. So did
13 you find and review the Keskin paper?
14   A. I found it and reviewed it, yes.
15   Q. Not provided to you by counsel; is that
16 right?
17   A. Can I see them both?
18   Q. Sure. Of course.
19   (Document was handed to the witness.)
20   THE WITNESS: I think I printed this
21 online, off of PubMed.
22 BY MR. ZELLERS:
23   Q. And my question is a little different.
24   Are these articles that you were made aware
25 of by plaintiffs' counsel, or are these articles that

Page 51

1 you found in any research that you did after being
2 retained in this matter?
3   A. I understand your question.
4   Yes, I researched and found these as I did
5 my PubMed search.
6   Q. All right. Latency, Exhibit 13.
7   (Exhibit No. 13 was marked for identification.)
8 BY MR. ZELLERS:
9   Q. You've got a couple of handwritten notes,
10 just a couple of articles in here. One is "The
11 latency period of mesothelioma among a cohort of
12 British asbestos workers (1978-2005)"; and also
13 "Latency Period for Malignant Mesothelioma" by
14 Dr. Lanphear, which is dated -- well, we'll have to
15 just let the record -- it was uploaded in 2016 by the
16 author.
17   Are these materials that you found in your
18 search and have put together, or are these articles
19 that were provided to you by counsel?
20   MS. O'DELL: Object to the form.
21   THE WITNESS: May I see that again?
22 BY MR. ZELLERS:
23   Q. Sure.
24   (Document was handed to the witness.)
25   THE WITNESS: I believe these are both

Page 52

1 articles that I identified in my literature search.
2 BY MR. ZELLERS:
3   Q. Did you find any articles on the latency
4 period of ovarian cancer in women?
5   A. The latency at the time of exposure to
6 asbestos or talcum powder?
7   Q. Yes.
8   A. I think it's clear that there has to be a
9 latency period, and it's probably very parallel, in my
10 opinion, to the latency period for mesothelioma and
11 many other cancers that requires decades of exposure
12 before one develops ovarian cancer.
13   Q. Can you be any more precise than "decades of
14 exposure"?
15   MS. O'DELL: Object to the form.
16   THE WITNESS: No more precise than
17 these papers that talk about the latency for
18 mesothelioma --
19 BY MR. ZELLERS:
20   Q. You believe --
21   A. -- which run the gamut from 22 to 32 years in
22 one paper and 20 to 40 years in another paper.
23   Q. You believe that the latency period for
24 ovarian cancer is the same as the latency period for
25 mesothelioma; is that right?

Page 53

1   MS. O'DELL: Object to the form.
2   THE WITNESS: I believe it should be
3 very close.
4 ///
5 ///
6   (Exhibit No. 14 was marked for identification.)
7 BY MR. ZELLERS:
8   Q. The last folder that you brought with you is
9 the -- is titled or captioned "Asbestos Fibers Talc
10 Longo, etc."
11   Is this also a folder that you prepared?
12   A. Yes, sir.
13   Q. You've got a number of handwritten notes and
14 calculations here; is that right?
15   MS. O'DELL: Object to the form.
16   THE WITNESS: I'm not sure it's
17 calculations. It's notes taken from the papers.
18 BY MR. ZELLERS:
19   Q. You cite and have brought with you a report,
20 Longo, January 15th, 2019.
21   Is that the updated report that was referred
22 to earlier?
23   A. That's my understanding.
24   Q. You've got, looks like, an exhibit from the
25 Hopkins deposition; is that right?

Page 54

1   A. Yes.
2   Q. You have an article by Blount, "Amphibole
3 Asbestos in Vermont Talc"; is that correct?
4   A. Yes.
5   Q. That's got an Imerys Bates number on it.
6   Is that where you obtained that document?
7   MS. O'DELL: Object to the form.
8   THE WITNESS: I obtained it from
9 counsel.
10 BY MR. ZELLERS
11   Q. And then you also have the Pier deposition
12 exhibit in your folder; is that right?
13   A. Yes.
14   Q. Have we now identified all of the materials
15 that you have reviewed and relied upon in formulating
16 your opinions in this matter?
17   A. Above and beyond these folders, the other
18 folders that we have here are included in my reliance.
19   Q. Your reliance list and your reference list;
20 is that right?
21   A. Yes.
22   Q. Exhibit A, just so we are complete here, is
23 your CV, or curriculum vitae, as of the time that your
24 report was published; is that right?
25   (Exhibit No. 15 was marked for identification.)

Page 55

1 BY MR. ZELLERS:
2   Q. And your report was published or provided and
3 signed in November of 2018?
4   And that's too many questions in one.
5   You attached an exhibit, Exhibit A, to your
6 report, which we have marked as Exhibit 5; is that
7 right?
8   MS. O'DELL: Is it -- Exhibit 15 is
9 the --
10   MR. ZELLERS: So Exhibit 15 is --
11 Deposition Exhibit 15 is a copy of Exhibit A to
12 Dr. Clarke-Pearson's report, which we marked as
13 Exhibit 5.
14 BY MR. ZELLERS:
15   Q. Number one, is that correct? Is this your
16 CV?
17   A. This is my CV at the time my report was
18 submitted.
19   Q. Is there a date on this curriculum vitae?
20   A. I don't believe so.
21   Q. Was it accurate and complete as of November
22 of 2018?
23   A. I'm just checking to see what my most recent
24 reference was in here. I try to keep it up to date.
25   Yes, I believe it was correct at the time

Page 56

1 that this was submitted in November 2018.
2   Q. Are there any updates to your curriculum
3 vitae that you believe in any way are relevant to the
4 opinions you're giving here today?
5   A. I understand. No, there's no -- nothing
6 relevant to add.
7   Q. I did not tell you at the beginning, but if
8 at any time you need to take a break and get up and
9 stretch, just tell me and we'll do that.
10   A. Okay.
11   MR. ZELLERS: Same goes for you as
12 well, Counsel.
13   MS. O'DELL: Thank you.
14 BY MR. ZELLERS:
15   Q. Did anyone assist you with your review and
16 research and preparation of your report in this matter
17 other than counsel?
18   A. No, sir.
19   Q. You were able to do the research that you
20 felt you needed to do to answer the questions that
21 were posed to you by counsel for the plaintiffs within
22 the 20 hours that are identified in your invoice,
23 Exhibit 2, between April 17th of 2017 and
24 November 4th of 2018?
25   A. That's what I billed for. As I sort of

Page 57

1 indicated earlier, I'm not very diligent on marking
2 down every minute or every hour that I spend. So
3 that's what I billed for. It's close to what time
4 I spent.
5   Q. That's your best estimate of the time that
6 you had spent on this matter through the preparation
7 of your report, which we marked as Exhibit 5; is that
8 right?
9   A. That's correct.
10   Q. When were you first asked to prepare a
11 report?
12   A. I'm not sure I can answer that question. It
13 was obviously after I'd been retained and after I'd
14 had the opportunity to review materials to be able to
15 formulate an opinion.
16   Q. Other than Ms. O'Dell and Dr. Thompson, what
17 other attorneys for the plaintiffs in the MDL talcum
18 powder litigation have you met with or communicated
19 with?
20   A. I met Ms. Brown yesterday for the first time.
21   Q. Anyone else?
22   A. No, sir.
23   Q. Do the -- strike that.
24   Does your invoice, Exhibit 2, approximate
25 the meetings and discussions that you had with

Daniel L. Clarke-Pearson, M.D.

Page 58

1 Dr. Thompson and Ms. O'Dell up and through the
2 production of your report in November of 2018?
3   MS. O'DELL: Objection. Form.
4   THE WITNESS: I believe so.
5 BY MR. ZELLERS:
6   Q. Since then, what other time have you spent
7 with the attorneys for plaintiffs relating to this
8 matter?
9   A. I've had one meeting, I believe in early
10 January, for an hour and a half or two --
11   Q. Was that an in-person meeting or --
12   A. Yes, it was in person.
13   Q. Was that here in Chapel Hill?
14   A. Yes.
15   Q. Was that with Ms. O'Dell and Dr. Thompson?
16   A. Yes.
17   Q. Anyone else?
18   A. No.
19   Q. Any other meetings that you've had with
20 counsel preparing for your deposition?
21   A. This past Saturday and Sunday.
22   Q. Did you meet with the three plaintiffs'
23 counsel who are here today?
24   A. Ms. O'Dell and Dr. Thompson on Saturday, and
25 Ms. Brown joined us on Sunday.

Page 59

1   Q. What amount of time did you spend, total, on
2 Saturday and Sunday with counsel preparing for the
3 deposition?
4   A. I'd estimate probably four to five hours on
5 Saturday and about five to six hours on Sunday.
6   Q. Anything else you did to prepare for your
7 deposition?
8   A. I reviewed a lot of materials here to be
9 really fresh on it. That's why you see these folders.
10   Q. Anything else you did to prepare for your
11 deposition?
12   A. I'm not sure I understand what else I might
13 do.
14   Q. Did you talk to anyone other than counsel for
15 plaintiffs?
16   A. I see. No, I didn't.
17   Q. Did you speak to any of your colleagues about
18 this?
19   A. No, sir.
20   Q. The total amount of time that you've spent,
21 you would approximate to be the 20 hours that are
22 reflected on Exhibit 2, plus an additional 60 hours up
23 until today when we started your deposition?
24   A. That would be my approximation, yes.
25   Q. Have you been retained in any other talcum

Page 60

1 powder proceeding, aside from the talcum powder MDL?
2   A. No.
3   Q. What percent of your professional time do you
4 spend working as a consultant?
5   A. With regard to medicolegal expert witness
6 work?
7   Q. Yes.
8   A. What percent? I'd say probably 5 percent in
9 this past year, less than that in the preceding
10 several years.
11   Q. What percent of your income is from
12 consulting on litigation matters?
13   A. None of my income.
14   Q. You receive no income as an expert witness
15 consultant on litigation?
16   A. No.
17   Q. Where does the money that you're billing for
18 your services as an expert witness in this case go?
19   A. The rules that we have at University of North
20 Carolina is that any revenue, if you will, from expert
21 witness work is considered clinical revenue and is
22 sent to the practice plan.
23   Q. Does your income, at least in part -- is it
24 determined by the income you bring into the
25 university?

Page 61

1   A. The compensation plan doesn't account for the
2 income we bring in.
3   Q. Your testimony is that doesn't matter what
4 grants you may bring in, it doesn't matter what expert
5 witness consulting you may do or what income you may
6 generate, it has no effect on your compensation; is
7 that right?
8   MS. O'DELL: Object to the form.
9   THE WITNESS: The Department of
10 Obstetrics & Gynecology at the University of North
11 Carolina, of which I'm the chair, the compensation
12 plan, the base salary is based on the AAMC median
13 income based on subspecialty.
14   So a maternal-fetal medicine physician,
15 based on their rank -- assistant, associate, and full
16 professor -- has a different median income than does a
17 gynecologic oncologist, but it's pegged to national
18 standards.
19 BY MR. ZELLERS:
20   Q. Is there any type of bonus or additional
21 compensation that someone in your department,
22 including yourself, can earn?
23   A. Yes.
24   Q. How or what are the factors in terms of bonus
25 compensation or additional compensation?

Daniel L. Clarke-Pearson, M.D.

Page 62

1   A. Clinical relative value units that are
2 generated by a faculty member that exceed the
3 60th percentile are then attributed to that faculty
4 member. The percent of the number of faculty members'
5 RVUs that are generated as a whole are then divided
6 out amongst the pot of money, if you will, that's
7 available for incentive distribution. And that amount
8 of money depends upon the department's overall
9 financial status.
10   Q. Do grants that are brought into the
11 university by members of your department have any
12 impact or part in this incentive distribution
13 calculation?
14   A. Yes.
15   Q. Do -- or strike that.
16   Does any income from litigation consulting
17 have a part in this incentive distribution?
18   A. No.
19   Q. Are you -- you are in charge of the
20 department; is that right?
21   A. I'm the chair of the department.
22   Q. Do you have to balance the books in terms of
23 money in and money out?
24   A. Yes, sir.
25   Q. Does income that you generate from litigation

Page 63

1 consulting help you balance the books of the
2 department?
3   A. Yes.
4   Q. The Deposition Exhibit 3, your list of
5 testimony that you've given in the past five years, is
6 that now accurate and complete?
7   A. Yes, sir.
8   Q. Have all of the testimonies you've given that
9 are listed on Exhibit 3, are those all deposition
10 testimony? Or have you testified at trial?
11   A. Let me take a look at them.
12   The Edmonson and Lee, I testified at trial.
13 Rappaport, I testified at trial. Pizzirusso, I
14 testified at trial. The latter two that I -- are just
15 depositions.
16   Q. Is it accurate you did not give deposition
17 testimony in Edmonson, Rappaport, and Pizzirusso?
18   A. No, that's not accurate.
19   Q. Well, should those depositions also be
20 included in this list of testimonies?
21   A. I don't know exactly what you asked for.
22 I -- this is either depositions or testimony that
23 I made in court.
24   Q. Did you give a deposition in Edmonson in the
25 past five years?

Page 64

1   A. Yes.
2   Q. Is that included in the disclosure that was
3 given to us today, Exhibit 3?
4   A. I considered it as deposition and trial
5 testimony.
6   Q. So there were two testimonies, both of which
7 you gave on December 12th of 2014; is that right?
8   A. No. That was probably when we submitted our
9 invoice. I got this information from my billing
10 department.
11   Q. So Edmonson really should be two testimonies;
12 is that right?
13   A. Yes. Deposition --
14   Q. And the deposition --
15   A. A deposition and trial testimony.
16   Q. And the date you've given here relates to
17 your invoice, not to when you provided the testimony?
18   A. I believe so.
19   Q. And the same answer with respect to
20 Rappaport. The date on Exhibit 3 doesn't relate to
21 when you provided the testimony; is that right?
22   A. That's right. And I had a deposition and
23 trial.
24   Q. And, lastly, with respect to the Pizzirusso
25 matter, the date doesn't relate to when you provided

Page 65

1 the testimony; correct?
2   A. That's correct.
3   Q. And it was actually a deposition and trial
4 testimony in those matters; is that right?
5   A. Yes.
6   Q. Have you ever been retained in a case
7 involving asbestos?
8   A. No.
9   Q. Have you ever been retained in a case
10 involving cosmetic products?
11   A. No, sir.
12   Q. Did you review any of the expert reports of
13 the other experts that have been retained by the
14 plaintiffs in the MDL talcum powder litigation?
15   MS. O'DELL: Other than Dr. Longo,
16 which he's testified to.
17   MR. ZELLERS: I'd like to hear it from
18 the doctor, but, yes, other than Dr. Longo.
19   THE WITNESS: I've read a lot of
20 things. Not many reports, so I don't recall exactly
21 if I -- may I ask counsel, since we've been working?
22 BY MR. ZELLERS:
23   Q. Well, no, because I really want it to be your
24 testimony. If you don't understand -- and I should
25 have told you this up front. If you have to guess or

Daniel L. Clarke-Pearson, M.D.

Page 66

1 speculate to answer my question, tell me you can't
2 answer it because it would call for a guess or
3 speculation.
4   A. Okay. I can't answer that.
5   Q. You don't recall, as you sit here, other than
6 Dr. Longo's updated report, reviewing any other expert
7 reports in this litigation; correct?
8   MS. O'DELL: Object to the form.
9   THE WITNESS: I reviewed Dr. Longo's
10 original report and now the updated report.
11 BY MR. ZELLERS:
12   Q. Other than those reports, at least as you sit
13 here, you don't have a memory of reviewing other
14 expert reports in this matter; is that right?
15   A. I don't recall.
16   Q. Do you recall reviewing any defense expert --
17 or strike that.
18   Do you recall reviewing any other expert
19 reports in any talcum powder litigation other than the
20 MDL?
21   A. No.
22   Q. Have you communicated about the litigation --
23 the MDL talcum powder litigation -- with anyone other
24 than plaintiffs' counsel?
25   A. I'm required to communicate that to the

Page 67

1 hospital counsel, and I have.
2   Q. Who is the hospital counsel?
3   A. Her name is Glenn -- G-L-E-N-N -- George.
4   Q. Does she work for the university directly or
5 is she in private practice, if you know?
6   A. She works for the University of North
7 Carolina Hospital as the head counsel.
8   Q. Have you communicated about talc as a cause
9 of ovarian cancer with anyone other than the
10 plaintiffs' counsel?
11   A. As it regards to this case?
12   Q. Yes, as it regards to this case and your
13 opinion that talcum powder used in the perineal region
14 by women is a cause of ovarian cancer.
15   A. I've communicated to the immediate past
16 president of the Society of Gynecologic Oncology that
17 I think that they should investigate and offer a
18 committee opinion on the topic.
19   Q. Who is the -- past president you said you
20 communicated with?
21   A. Past president.
22   Q. Who is that?
23   A. Her name is Laurel Rice, R-I-C-E.
24   Q. When did you have that communication with
25 Dr. Rice?

Page 68

1   A. Sometime after I formed my opinion. I'm not
2 sure. I'm in communication with Dr. Rice quite often.
3 She's a friend of mine.
4   Q. Was it before or after you prepared your
5 report --
6   A. It was after my report.
7   Q. So sometime after November --
8   A. 16th.
9   Q. -- 16th of 2018; is that right?
10   A. Yes.
11   Q. Any other communication you've had with
12 anyone other than counsel for plaintiffs regarding
13 your opinion that talc is a cause of ovarian cancer?
14   A. No.
15   Q. Have you reviewed any deposition or trial
16 testimony from any of the talcum powder cases?
17   A. Yes. I'm blanking on her name. The GYN
18 oncologist, Judy -- one of the experts on the
19 plaintiffs' side that --
20   Q. Judy Wolf?
21   A. Yeah, Judy Wolf.
22   Q. Do you know Dr. Wolf?
23   A. I've met her once.
24   Q. Have you had any discussions with her about
25 the subject matter of your opinions in this case with

Page 69

1 Dr. Wolf?
2   A. I've had no communication with Dr. Wolf
3 whatsoever.
4   Q. You reviewed her deposition transcript in
5 preparation for today; correct?
6   A. Yes.
7   Q. Any other deposition transcripts or trial
8 transcripts in the talcum powder litigation or any
9 talcum powder case that you have reviewed?
10   A. Reviewed -- I can't remember the name --
11 Pinkerton, maybe. It was a toxicologist that had a
12 deposition.
13   Q. Do you remember the name or do you -- did you
14 know this toxicologist?
15   A. I don't know the toxicologist. I think the
16 name was Pinkerton.
17   Q. Any other deposition transcripts or trial
18 transcripts that you have reviewed?
19   A. No, sir.
20   Q. Were the transcripts of Dr. Wolf and
21 Pinkerton, the toxicologist, provided to you by
22 counsel for the plaintiffs?
23   A. Yes.
24   Q. Did you request any information or material
25 from counsel for the plaintiffs that was not provided

Daniel L. Clarke-Pearson, M.D.

Page 70

1 to you?
2   A. No. I think everything was provided to me
3 that I requested.
4   Q. In your report and in one of your file
5 folders, you have exhibits from the deposition of John
6 Hopkins. And let me rephrase that. You have an
7 exhibit from a witness by the name of John Hopkins.
8 Are you aware of that?
9   A. Yes.
10   Q. Who is Mr. Hopkins?
11   A. I've been -- it's my understanding -- and
12 I may be wrong -- that he is a former employee of
13 Johnson & Johnson.
14   Q. Do you know what he did for Johnson &
15 Johnson?
16   A. I believe somehow he was involved with
17 testing of talcum powder to evaluate for products such
18 as fibrous talc and asbestos.
19   Q. Do you know anything else that Mr. Tom --
20 Mr. Hopkins did for Johnson & Johnson?
21   A. No.
22   Q. Did you review or read his deposition?
23   A. I did not.
24   Q. Do you know who Julie Pier is?
25   A. Vaguely.

Page 71

1   Q. Who is Julie Pier?
2   A. My understanding is that she has also done
3 testing on Johnson & Johnson products.
4   Q. Do you know where she works or by whom she is
5 employed?
6   A. No.
7   Q. Did you read her deposition transcript?
8   A. No.
9   Q. Have you reviewed any other exhibits to the
10 deposition of John Hopkins?
11   A. No, sir.
12   Q. Have you reviewed any other exhibits to the
13 deposition of Julie Pier?
14   A. No.
15   Q. Is it your practice outside of litigation to
16 rely on isolated exhibits from deposition testimony?
17   MS. O'DELL: Object to the form.
18   THE WITNESS: I think sometimes if
19 they're meaningful, yes.
20 BY MR. ZELLERS:
21   Q. Have you ever, in any of the peer-reviewed
22 publications that are listed in Exhibit A, cited to
23 isolated exhibits from deposition testimony of
24 depositions that you didn't read?
25   MS. O'DELL: Object to the form.

Page 72

1   THE WITNESS: I'm sorry. You're asking
2 me about peer-reviewed publications?
3 BY MR. ZELLERS:
4   Q. Yes, and whether or not you have ever relied
5 upon isolated exhibits provided to you by counsel from
6 depositions that you have never read as support for
7 any of your peer-reviewed publications.
8   A. In a peer-reviewed publication, one on
9 occasion will cite a personal communication from a
10 colleague or an expert.
11   Q. Can you answer my question?
12   A. "In a peer-reviewed publication, one on
13 occasion will cite a personal communication" -- okay.
14 So your question was -- all right.
15   So in my peer-reviewed publications, I would
16 say the answer is no.
17   Q. What is the difference between the references
18 which are at the end of your report that we marked as
19 Exhibit 5 and the list of additional materials which
20 we marked as Deposition Exhibit 6 and you included as
21 Exhibit B to your report?
22   A. Those are additional materials that
23 I reviewed in formulating my opinion, but I felt that
24 they didn't need to be included in my report.
25   Q. Were the references that you listed in your

Page 73

1 report, Exhibit 5, the key primary materials that
2 you're relying on?
3   MS. O'DELL: Object to the form.
4   THE WITNESS: I think that's fair to
5 say, yes.
6 BY MR. ZELLERS:
7   Q. If you go to Exhibit 6 -- could you find that
8 in front of you. This, again, is Exhibit B to your
9 report. Go to page 11.
10   And you see, starting at the bottom of page
11 11 carried over to page 12, there are a number of
12 documents that begin with "Imerys" and then have a
13 number following them.
14   Do you see that?
15   A. Yes.
16   Q. Did you rely on those documents in forming
17 your opinions?
18   A. I reviewed them.
19   Q. Can you identify for us here what those
20 documents are?
21   A. I would have to go to the books to review
22 them.
23   Q. Do you know how those documents were
24 compiled?
25   A. They were supplied by counsel.

Daniel L. Clarke-Pearson, M.D.

Page 74

1   Q. Turning to page 13, there's a series of
2 documents that begin with "J&J" followed by numbers.
3   Do you see that?
4   A. Yes.
5   Q. Did you rely on those documents in forming
6 your opinions?
7   A. I reviewed them, and they probably served as
8 part of my overall opinion; but I'm not referencing
9 them per se in my report.
10   Q. Can you identify or tell us what those
11 documents are?
12   A. These were internal documents from J&J.
13 I don't recall specifically what each one of these
14 numbers represent.
15   Q. Do you know how they were compiled?
16   A. They were provided to me by counsel.
17   Q. Plaintiffs' counsel provided you with these
18 select company documents that you have identified in
19 your additional materials list; is that right?
20   A. Yes.
21   MS. O'DELL: Object to the form.
22 BY MR. ZELLERS:
23   Q. Were you provided with any documents of
24 either Imerys or J&J by counsel for plaintiffs that
25 you did not include or list in your additional

Page 75

1 materials-considered list?
2   A. No. I believe I've listed everything that we
3 saw.
4   Q. Based upon -- well, strike that.
5   Did you review each of these documents of
6 Imerys and J&J that are identified in your
7 materials-reviewed list?
8   MS. O'DELL: Objection. Asked and
9 answered.
10   THE WITNESS: Yes.
11 BY MR. ZELLERS:
12   Q. Based upon that review, did you ask
13 plaintiffs' counsel if there were any additional
14 documents or documents that might put in context the
15 documents that were selected by plaintiffs' counsel
16 for you to review?
17   MS. O'DELL: Object to the form.
18   THE WITNESS: No, I didn't ask for
19 that.
20 BY MR. ZELLERS:
21   Q. Outside of your work in litigation, do you,
22 with respect to your scientific publications and work,
23 rely on small subsets of internal company documents?
24   MS. O'DELL: Object to the form.
25   THE WITNESS: I believe this is the

Page 76

1 first time I've been shown internal documents in a
2 litigation.
3 BY MR. ZELLERS:
4   Q. Do you have any knowledge as to what
5 percentage of the internal documents that have been
6 produced in this litigation were actually provided to
7 you and appear in your materials-considered list,
8 Exhibit 6 to this deposition?
9   MS. O'DELL: Object to the form.
10   THE WITNESS: I do not.
11 BY MR. ZELLERS:
12   Q. Is it fair to say, Dr. Clarke-Pearson, that
13 the only company documents that you reviewed -- either
14 Imerys or Johnson & Johnson -- are the ones that were
15 hand-selected by plaintiffs' lawyers and provided to
16 you?
17   A. Yes, that's fair to say.
18   Q. Do you agree, based upon your experience and
19 the studies that you've reviewed, that most women who
20 used talcum powder in their perineal region begin that
21 use before age 30?
22   MS. O'DELL: Object to the form.
23   THE WITNESS: I believe that's
24 reasonable. I'm not aware of any data that
25 specifically says that.

Page 77

1 BY MR. ZELLERS:
2   Q. Well, the Cramer 2016 paper actually goes
3 through and lists out the age for the folks that were
4 included in that study first used genital powder. Is
5 that generally familiar to you?
6   A. I can pull the paper if we're going to need
7 to discuss it more, but...
8   Q. Well, my question is -- and you can decide if
9 you need to pull the paper. But do you agree that,
10 based upon your review of the literature, your
11 personal experience, that the vast majority of women
12 who use talcum powder in their perineal region begin
13 that use before the age of 30?
14   If you need to take a look at the Cramer
15 paper, go to page 336. This is Cramer 2016, Table 1.
16   A. So --
17   Q. I think it's a simple question --
18   A. Probably so.
19   So can you restate the question? I've lost
20 it on the screen.
21   Q. Sure.
22   Do you agree that most women who use talcum
23 powder in their perineal region begin that use before
24 age 30?
25   A. Yes.

Daniel L. Clarke-Pearson, M.D.

Page 78

1   Q. Do you agree that, on average, women who use
2 talcum powder in their perineal region continue that
3 use for over 20 years?
4   A. Yes.
5   Q. It's your opinion that talcum powder causes
6 ovarian cancer; is that right?
7   A. Yes, sir.
8   Q. What are the other causes of ovarian cancer?
9   A. We can talk about risk factors --
10   Q. No, I don't want to talk about risk factors.
11 You have identified talcum powder as a causative
12 factor in ovarian cancer; is that right?
13   A. Right.
14   Q. That's different than being a risk factor for
15 ovarian cancer; is that right?
16   MS. O'DELL: Object to the form.
17   THE WITNESS: I'm not sure that's true.
18 BY MR. ZELLERS:
19   Q. Well, is it your opinion that ovarian cancer
20 is caused by talcum powder or that talcum powder is a
21 risk factor for ovarian cancer?
22   A. Ovarian cancer is caused by talcum powder.
23   Q. What other causes of ovarian cancer are
24 there, in your opinion?
25   A. Fair enough.

Page 79

1   Age, lack of exposure to birth control
2 pills, lack of being pregnant -- so nulliparity --
3 obesity, women that have had pelvic inflammatory
4 disease, women who use a nonhormonal-producing
5 intrauterine device, women who have gene mutations for
6 BRCA1, 2, or Lynch syndrome.
7   There are probably others; but, off the top
8 of my head, I think that's a fairly complete list.
9   Q. Each of the items that you have mentioned, in
10 your opinion, those are causes of ovarian cancer; is
11 that right?
12   A. Yes.
13   Q. What is the difference between a risk factor
14 and a cause?
15   A. They're virtually the same. A risk factor
16 describes a cause. It does not affect every woman
17 that has that risk factor.
18   Q. Is that true for all of the risk factors that
19 you just identified?
20   A. Yes.
21   Q. Is that true for talcum powder?
22   A. Yes.
23   Q. What makes a factor cross the line from being
24 a risk factor to being a cause?
25   A. Well, I think that the risk factor is the

Page 80

1 cause, but the cause doesn't -- but the risk factor
2 doesn't cause the cancer in every instance.
3   Q. Talcum powder is a risk factor for ovarian
4 cancer; is that right?
5   A. And it causes ovarian cancer.
6   Q. Every factor that you identified for us --
7 age, pelvic inflammatory disease, obesity -- those are
8 all risk factors for ovarian cancer and, in your
9 opinion, causes of ovarian cancer; is that right?
10   A. Yes.
11   Q. If a study shows a statistically significant
12 relationship between a risk factor and a disease, is
13 that enough for the factor to be classified as a
14 cause?
15   A. In my opinion, yes.
16   Q. Just takes one study; is that right?
17   MS. O'DELL: Object to the form.
18   THE WITNESS: No. Now we're talking
19 about the totality of the evidence, and nearly all of
20 those -- all those risk factors that I described to
21 you that are causative for ovarian cancer, including
22 talcum powder, there's more than just one study.
23 BY MR. ZELLERS:
24   Q. Let me ask my question again because I may
25 not have been clear.

Page 81

1   If a study shows a statistically significant
2 relationship between a risk factor and a disease, is
3 that enough for the factor to be classified as a
4 cause?
5   A. I see what you're saying.
6   So, no, one study is not sufficient, in my
7 opinion.
8   Q. Other than your discussion with Dr. Rice
9 sometime after November 16th of 2018, what have you
10 done to alert the medical community about the
11 relationship between talcum powder and ovarian cancer?
12   MS. O'DELL: Object to the form.
13   THE WITNESS: That's all I've done
14 right now.
15 BY MR. ZELLERS:
16   Q. What was your methodology for concluding that
17 talcum powder causes ovarian cancer?
18   A. All right. So then we get into what
19 I describe as my methods to come to this conclusion.
20 And I was asked by counsel to form an opinion one way
21 or the other.
22   To do that, I used very similar techniques
23 that I use in doing peer-reviewed publications, of
24 which I have over 250 and over 50 book chapters.
25 I need to research the literature.

Daniel L. Clarke-Pearson, M.D.

Page 82

1   In this case, I used a PubMed search.
2 I also used a Google search. And I reviewed a number
3 of textbooks. In my PubMed search, many times there
4 were references that then I would turn to and also
5 pull up to review; and that's where many of these
6 publications over here in these binders come from.
7   As I then start working my way through it,
8 we start -- you know, in medicine, I would call it
9 evidence-based medicine. In this particular
10 circumstance, Bradford Hill criteria are used to come
11 to a conclusion. And I have my Bradford Hill summary
12 in the back of my -- at the end of my report to show
13 you how I came to my conclusions that talcum powder
14 causes ovarian cancer.
15   Q. Anything else that you did in terms of your
16 methodology for concluding that talcum powder causes
17 ovarian cancer?
18   A. I, you know, of course, in looking at
19 publications come to try to put some weight on the
20 publications, whether this is something that should be
21 given more weight or less weight.
22   I don't have a scoring system per se, but
23 evaluating the size of the study, the statistical
24 analysis, the study design, the credibility of the
25 author, the quality of the journal that the

Page 83

1 publication is printed in are all things that come to
2 my -- fit into my evaluation and help me come to my
3 conclusion.
4   Q. Anything else?
5   A. In the end, it's a matter of the totality of
6 what I've reviewed to bring forward my opinion based
7 on the Bradford Hill criteria.
8   Q. Anything else?
9   A. Not that I'm aware of except for my own
10 personal experience as a gynecologic oncologist for
11 nearly 40 years. And I've harkened back several times
12 already to my early training and then subsequent to
13 that.
14   Q. Did you follow this same methodology with
15 regard to the other question that you addressed,
16 whether or not there was a biologic mechanism by which
17 talcum powder could cause ovarian cancer?
18   A. Yes, sir.
19   Q. Do you believe that the standard for proving
20 causation in the medical literature is the same as the
21 one that applies in litigation?
22   MS. O'DELL: Object to the form.
23   THE WITNESS: I think that we use --
24 whether you want to call it Bradford Hill or whether
25 we want to call it evidence-based medicine, those are,

Page 84

1 I think, pretty much interchangeable terms.
2   I think in evidence-based medicine probably
3 fits more into my clinical practice, and it's my
4 understanding Bradford Hill fits more into litigation.
5 BY MR. ZELLERS:
6   Q. Try to answer my question if you can.
7   Do you believe that the standard for proving
8 causation in the medical and scientific literature is
9 the same as the one that applies in litigation?
10   MS. O'DELL: Object to the form. Asked
11 and answered.
12   THE WITNESS: I believe so.
13 BY MR. ZELLERS:
14   Q. Is it generally known among gynecological
15 oncologists that talcum powder causes ovarian cancer?
16   A. Not until recently. I think I referred to a
17 tipping point that's happening right now that will
18 make more gynecologic oncologists aware of the
19 problem.
20   Q. At least as of now, though, the answer would
21 be no based upon your experience; correct?
22   A. My experience at the moment is that many
23 gynecologic oncologists are starting to suspect that
24 there is an association and that talcum powder causes
25 ovarian cancer based on the literature and then also,

Page 85

1 importantly, on what the news media has been
2 reporting.
3   Q. What was your methodology for focusing on
4 certain studies and excluding or not addressing other
5 studies in your review?
6   MS. O'DELL: Object to the form.
7   THE WITNESS: Well, I think I tried to
8 answer that before. I was trying to put a weight to
9 those studies that are more or less strong, if you
10 will, and -- and others that are there but really
11 don't have any input or bearing on my decision.
12 BY MR. ZELLERS:
13   Q. You do not discuss or address the cohort
14 studies in your report; is that right?
15   A. That's true.
16   MS. O'DELL: Object to the form.
17 BY MR. ZELLERS:
18   Q. Would you agree that, if you had only looked
19 at the cohort studies in this case, that you would not
20 have been able to opine that talcum powder causes
21 ovarian cancer?
22   MS. O'DELL: Object to the form.
23   THE WITNESS: Exactly why I tried to do
24 a full literature search and included case-control
25 studies.

Daniel L. Clarke-Pearson, M.D.

Page 86

BY MR. ZELLERS:

1 Q. You believe -- well, strike that.

3 You have published a number of articles on
4 ovarian cancer; is that right?

5 A. I believe so.

6 Q. In any of those articles, have you published
7 your theory that baby powder causes ovarian cancer?

8 MS. O'DELL: Object to the form.

9 THE WITNESS: The intention of those
10 articles was not to address causation or risk factors.

11 BY MR. ZELLERS:

12 Q. Is the answer no, that you have not, at least
13 in those publications, discussed your theory that baby
14 powder causes ovarian cancer?

15 MS. O'DELL: Object to the form.

16 THE WITNESS: Those papers were not
17 intended to discuss risk factors associated with
18 talcum powder, so the answer is no.

19 BY MR. ZELLERS:

20 Q. Have you conducted any tests or experiments
21 to confirm your theory that talc migrates from the
22 perineum to the ovaries?

23 MS. O'DELL: Object to the form.

24 THE WITNESS: It's my opinion -- and
25 this is not a theory -- that it's well established in

Page 87

1 the gynecologic community that talc can migrate along
2 with other particles from the perineum to the ovarian
3 surface and fallopian tube.

4 BY MR. ZELLERS:

5 Q. Try and answer my question if you can.

6 Have you, Dr. Clarke-Pearson, conducted any
7 tests or experiments to confirm the theory that talc
8 migrates from the perineum to the ovaries?

9 MS. O'DELL: Object to the form.

10 THE WITNESS: No, I have not.

11 BY MR. ZELLERS:

12 Q. Have you, Dr. Clarke-Pearson, conducted any
13 tests or experiments to confirm your theory that talc
14 causes cancer via inflammation?

15 MS. O'DELL: Object to the form.

16 THE WITNESS: It's not my theory; it's
17 my opinion that talc causes ovarian cancer through
18 inflammation.

19 I have not done any studies to confirm my
20 opinion.

21 BY MR. ZELLERS:

22 Q. Can you identify a single article that
23 identifies inflammation anywhere in a woman's
24 reproductive tract resulting from external genital
25 talc application?

Page 88

1 MS. O'DELL: Mike, after
2 Dr. Clarke-Pearson answers this question, we've been
3 going about an hour and 50 minutes. If we could take
4 a break, that would be great.

5 MR. ZELLERS: That's fine. I've got
6 one more after this, and then would be glad to take a
7 break.

8 BY MR. ZELLERS:

9 Q. Dr. Clarke-Pearson, can you answer that?

10 A. I thought I had a folder on inflammation
11 here. I don't think you put it under your pile. But,
12 at any rate, I think I have seen evidence that talc
13 can cause inflammation in the ovary.

14 Q. Let me ask my question again.

15 Can you identify a single article that
16 identifies inflammation anywhere in a woman's
17 reproductive tract resulting from external genital
18 talc application?

19 MS. O'DELL: Object to the form.

20 THE WITNESS: I don't believe so, that
21 I can quote for you right now.

22 BY MR. ZELLERS:

23 Q. Can you cite a single study, animal or human,
24 that traces externally applied talc up through the
25 reproductive tract to the ovaries?

Page 89

1 A. I think that's well accepted, as I said, in
2 the gynecologic community, that the vagina is open to
3 the outside world, if you will, there's no lid at the
4 opening of the vagina, and that particles of talc can
5 migrate from the vulva and perineum up through the
6 uterus and onto the ovaries.

7 Q. Now I need you to answer my question. Do you
8 need me to repeat it?

9 MS. O'DELL: Well, Counsel, won't you
10 be courteous of the witness. He answered your
11 question. You may not have liked the answer. And you
12 happy to ask another question.

13 MR. ZELLERS: No, he did not answer my
14 question.

15 MS. O'DELL: He did answer your
16 question.

17 MR. ZELLERS: The record will reflect
18 he did not. And I think both of us, all of us, are
19 being cordial and professional.

20 If, at any time, Dr. Clarke-Pearson, you
21 don't think I'm being professional, let me know.
22 Okay?

23 THE WITNESS: Sure.

24 BY MR. ZELLERS:

25 Q. My specific question to you is can you cite

Daniel L. Clarke-Pearson, M.D.

Page 90

1 any study, animal or human, that traces externally
2 applied talc up through the reproductive tact to the
3 ovaries?
4 　　　　MS. O'DELL: Object to the form.
5 　　　　THE WITNESS: So by study, you mean a
6 peer-reviewed publication?
7 BY MR. ZELLERS:
8 　　Q. Yes.
9 　　A. I cannot.
10 　　　　MR. ZELLERS: Let's take a break.
11 　　　　THE VIDEOGRAPHER: Going off the record
12 at 10:50 a m.
13 　　(Recess taken from 10:50 a m. to 11:04 a.m.)
14 　　　　THE VIDEOGRAPHER: Back on record at
15 11:04 a m.
16 BY MR. ZELLERS:
17 　　Q. Dr. Clarke-Pearson, do you treat women who
18 have ovarian cancer and other gynecological disease?
19 　　A. I've treated hundreds of women with ovarian
20 cancer, put them through radical surgical procedures,
21 including bowel resections and removing their spleen
22 to get their cancer out. I've given them
23 chemotherapy. We've had some successes. I've taken
24 care of a lot of patients throughout the remainder of
25 their life as they died from ovarian cancer.

Page 91

1 　　　　So to answer your question, yes.
2 　　Q. Do you also counsel women who are at high
3 risk for ovarian cancer?
4 　　　　MS. O'DELL: Object to the form.
5 　　　　THE WITNESS: Yes.
6 BY MR. ZELLERS:
7 　　Q. Ovarian cancer is a complex disease; correct?
8 　　A. Cancer, in general, is a complex disease.
9 I wish we knew more about it.
10 　　Q. No one knows for sure how ovarian cancer
11 develops; is that right?
12 　　　　MS. O'DELL: Object to the form.
13 　　　　THE WITNESS: I think we have some
14 strong opinions based on scientific research, and we
15 continue to research further in terms of the genetics
16 and mutations that go along with developing ovarian
17 cancer.
18 BY MR. ZELLERS:
19 　　Q. Is it true that no one knows for sure how
20 ovarian cancer develops?
21 　　　　MS. O'DELL: Object to the form.
22 　　　　THE WITNESS: I guess no one knows for
23 sure.
24 BY MR. ZELLERS:
25 　　Q. You refer in your report to there being

Page 92

1 several theories as to the origin of ovarian cancer;
2 is that right?
3 　　　　MS. O'DELL: Object to the form.
4 　　　　THE WITNESS: Yes.
5 BY MR. ZELLERS:
6 　　Q. Do you agree that, although some risk
7 factors, like age or BRCA genetic mutations have been
8 identified, it's impossible to say for sure what the
9 cause of ovarian cancer was for any individual woman?
10 　　　　MS. O'DELL: Object to the form.
11 　　　　THE WITNESS: Well, we know that the
12 cause is a genetic mutation that allows the ovarian
13 cancer -- that ovarian cell that was normal to become
14 a malignant cell and loses its regulation and growth.
15 BY MR. ZELLERS:
16 　　Q. Do you agree, though, that it is impossible
17 to say for sure what the cause of ovarian cancer was
18 for any individual woman?
19 　　　　MS. O'DELL: Object to the form.
20 　　　　THE WITNESS: The cause is always a
21 gene mutation.
22 BY MR. ZELLERS:
23 　　Q. Is it your testimony that you are able to
24 identify the cause of ovarian cancer in all cases?
25 　　　　MS. O'DELL: Object to the form.

Page 93

1 　　　　THE WITNESS: I can't identify the gene
2 mutation in all cases, no.
3 BY MR. ZELLERS:
4 　　Q. Is it impossible to say for sure what gene
5 mutation or other cause of ovarian cancer was for any
6 individual woman?
7 　　　　MS. O'DELL: Object to the form.
8 　　　　THE WITNESS: In some individual women,
9 we can identify the cause, for example, the mutation
10 of the BRCA1 and 2 gene. We can also do genetic
11 profiling more and more these days, identifying a
12 number of gene mutations that then lead to the
13 malignancy.
14 BY MR. ZELLERS:
15 　　Q. Other than BRCA1 and 2, do you agree that it
16 is impossible to say for sure what the cause of
17 ovarian cancer was for any individual woman?
18 　　　　MS. O'DELL: Object to the form.
19 　　　　THE WITNESS: There are more gene
20 mutations than BRCA 1 and 2. There's PD1 and others
21 that I don't have off the top of my head that are now
22 being identified.
23 BY MR. ZELLERS:
24 　　Q. Other than when a specific gene mutation can
25 be identified, is it impossible to say for sure what

Daniel L. Clarke-Pearson, M.D.

Page 94

1 the cause of ovarian cancer was for any individual
2 woman?
3   MS. O'DELL: Object to the form.
4   THE WITNESS: In -- to answer your
5 question, what I think I understand your question
6 being, if we can't identify a gene mutation, then we
7 don't know what caused it. Is that what you're asking
8 me?
9 BY MR. ZELLERS:
10   Q. Yes.
11   A. Then the answer would be, yes, we don't know.
12   Q. In your practice, do you diagnose what caused
13 your patients' ovarian cancer?
14   A. We do genetic profiling, as is a relatively
15 new approach to trying to approach causes, and also
16 personalized treatment for patients with ovarian
17 cancer.
18   Q. Other than genetic profiling, in your
19 practice do you diagnose what caused your patients'
20 ovarian cancer?
21   MS. O'DELL: Object to the form.
22   THE WITNESS: We don't. There's no --
23 I don't think anybody can.
24 BY MR. ZELLERS:
25   Q. In your practice, do you tell your patients

Page 95

1 what caused their ovarian cancer other than with
2 respect to genetic profiling?
3   MS. O'DELL: Object to the form.
4   THE WITNESS: No.
5 BY MR. ZELLERS:
6   Q. Have you ever given any presentation on the
7 relationship between talcum powder and ovarian cancer?
8   A. No.
9   Q. Have you ever spoken at a conference or
10 meeting of the American College of Obstetricians and
11 Gynecologists, or ACOG, about the relationship between
12 talcum powder and ovarian cancer?
13   A. Not that I recall.
14   Q. Have you ever spoken at a conference or
15 meeting of the Society of Gynecologic Oncology, or
16 SGO, about the relationship between talcum powder and
17 ovarian cancer?
18   A. No.
19   Q. Does your institution, the University of
20 North Carolina, advise women that perineal use of
21 talcum powder causes ovarian cancer?
22   A. Well, again, back to my point of the tipping
23 point in this whole discussion. And so at this
24 juncture, we are considering adding that to our
25 patient intake form, to ask for that information, and

Page 96

1 then also advise.
2   Q. As of today, it's not part of the patient
3 intake form; is that right?
4   A. As of today, no.
5   Q. As of today, the University of North Carolina
6 and the department that you chair do not advise women
7 that perineal use of talcum powder causes ovarian
8 cancer; correct?
9   MS. O'DELL: Object to the form.
10   THE WITNESS: That's correct.
11 BY MR. ZELLERS:
12   Q. Do you teach residents about talc as a
13 potential risk factor?
14   A. It is listed as a potential risk factor
15 today, and I think in the very near future it will be
16 considered a risk factor and a causative factor.
17   Q. When did you first start doing that, teaching
18 residents about talc as a potential risk factor?
19   A. Well, I think it's been in the literature for
20 a good while as a potential risk factor.
21   Q. My question is when did you first begin
22 teaching residents about talc as a potential risk
23 factor?
24   A. I think from the time that I was starting to
25 teach residents in 1975 -- well, I was a resident in

Page 97

1 '75 -- 1979 when I finished my residency and started
2 teaching residents.
3   Q. Do you today ask any of your own patients if
4 they used talcum powder as a routine screening
5 question?
6   A. I think that would be very inappropriate for
7 a woman who has advanced ovarian cancer to try to find
8 and cause her to feel guilt that she did something to
9 cause ovarian cancer. My situation is one of trying
10 to take care of women that have ovarian cancer.
11   Q. Have you ever told a patient that talcum
12 powder caused her ovarian cancer?
13   A. No.
14   Q. Have you ever recommended increased screening
15 or monitoring for ovarian cancer based on a patient's
16 prior use of talcum powder products?
17   A. Not yet.
18   Q. Have you ever recommended that a patient who
19 had a history of using talcum powder undergo
20 prophylactic surgery to remove the fallopian tubes or
21 ovaries because of her talcum powder use?
22   A. I think that is likely to become a discussion
23 in the near future, and we would have to balance the
24 risks of surgery versus the risks of developing
25 ovarian cancer.

Daniel L. Clarke-Pearson, M.D.

Page 98

1   Q. As of today, you have not; is that right?
2   A. That's correct.
3   Q. Have you ever asked your patients about their
4 exposure to asbestos in the course of taking their
5 medical histories?
6   A. No.
7   Q. Are you familiar with screenings for asbestos
8 exposure?
9   A. I'm not familiar with that.
10   Q. Do you ask your patients about their
11 occupational history?
12   A. I often -- yes, most of the time I find out
13 what the patient does outside the home.
14   Q. Do you ask your patients about the
15 occupational history of their parents?
16   A. I do not.
17   Q. Do you ask your patients about their spouse's
18 occupational history?
19   A. Sometimes.
20   Q. Do you ask what kind of buildings your
21 patients have either lived in or do live in?
22   A. No.
23   Q. Do you ask about the kind of buildings that
24 your patients either work in or have worked in?
25   A. Have not.

Page 99

1   Q. In 1993 you coauthored an article on the
2 mutations of the p53 gene and ovarian cancer; is that
3 right?
4   A. I believe so. I was a coauthor.
5   Q. That study concluded that p53 mutations in
6 ovarian cancer arise because of spontaneous errors in
7 DNA synthesis and repair rather than direct
8 interaction with -- strike that -- rather than the
9 direct interaction of carcinogens with DNA; is that
10 right?
11   MS. O'DELL: He needed --
12   THE WITNESS: I would have to see that
13 paper. 1993 was a long time ago. It was kind of our
14 lab. And I was not in the lab, but I was a coauthor.
15   MR. ZELLERS: Deposition Exhibit 16 is
16 the paper on which you were an author. First named
17 author was Kohler.
18   (Exhibit No. 16 was marked for identification.)
19 BY MR. ZELLERS:
20   Q. Take just a quick look at that, and I have a
21 specific question for you.
22   This is your paper that you were a coauthor
23 on back in 1993; is that right?
24   A. Allow me to read this a little bit more.
25   Q. Sure.

Page 100

1   A. All right. I think I can answer this. This
2 is a long time ago.
3   Q. As -- and let me just repeat my question, and
4 I'm specifically looking at the statement toward the
5 bottom of the third column on page 1 of the
6 publication.
7   The study concluded that p53 mutations in
8 ovarian cancer arise because of spontaneous errors in
9 DNA synthesis and repair rather than the direct
10 interaction of carcinogens with DNA; is that right?
11   A. That's what it reads.
12   Q. That would be inconsistent with the idea that
13 exposure to talcum powder causes errors in DNA
14 synthesis and repair that lead to cancer; is that
15 right?
16   MS. O'DELL: Object to the form.
17   THE WITNESS: No, that's not -- that's
18 not correct.
19 BY MR. ZELLERS:
20   Q. Why is that not correct?
21   A. So the inflammatory response of the ovarian
22 epithelium to talcum powder then leads to gene
23 mutations, and there is mounting evidence that that's
24 happening in work that's being written and presented
25 by Dr. Saed in particular.

Page 101

1   Q. Does your paper -- the 1993 paper -- discuss
2 inflammation?
3   A. No. That wasn't part of the question that
4 was being pursued in this laboratory investigation.
5   Q. Your paper in 1983 [sic] states that
6 (as read):
7   "Consistent with data from
8   epidemiologic studies that failed
9   to demonstrate a convincing
10   relationship between ovarian
11   cancer and exposure to
12   environmental carcinogens."
13   Is that right?
14   MS. O'DELL: Object to the form.
15 BY MR. ZELLERS:
16   Q. And I'm looking again at the first page of
17 your article at the bottom -- or right above the line
18 in the third column.
19   A. You've read that correctly. I would have to
20 reread this paper -- it's more than 20 years old --
21 because I'm not continue -- I'm not currently aware of
22 the investigation that we did looking at carcinogens.
23   Q. In 2009, you published an article entitled
24 "Screening for Ovarian Cancer." Is that right?
25   A. I'd have to see the article.

Daniel L. Clarke-Pearson, M.D.

Page 102

1   MR. ZELLERS: We'll mark your 2009
2 article as Deposition Exhibit 17.
3   (Exhibit No. 17 was marked for identification.)
4   THE WITNESS: Yes. Okay.
5 BY MR. ZELLERS:
6   Q. This is an article that you authored; is that
7 right?
8   A. Yes, it was printed in The New England
9 Journal. I was invited to write this clinical review.
10   Q. This is an article that is captioned
11 "Screening for Ovarian Cancer." Is that right?
12   A. Yes.
13   Q. This is many years before you were retained
14 by Dr. Thompson and plaintiffs' counsel in the talcum
15 powder litigation; is that right?
16   A. Yes.
17   Q. In this article, you discussed risk factors
18 for ovarian cancer. And I'm looking at the second
19 paragraph on page 1.
20   A. The first page of -- page 170?
21   Q. Yes. And my question, specifically, is you
22 only discussed in this article the risk factors of
23 family history of ovarian or breast cancer and the
24 BRCA genetic mutations; is that right?
25   MS. O'DELL: Object to the form.

Page 103

1   THE WITNESS: That's what appears to
2 be, yes.
3 BY MR. ZELLERS:
4   Q. You did not mention talcum powder in this
5 article; is that right?
6   A. It appears I didn't mention several other
7 risk factors. That wasn't the intent of this article.
8   Q. Well, in July of 2014, you appeared on a FOX
9 News station to discuss ovarian cancer; do you
10 remember that?
11   A. Vaguely.
12   Q. That was before you were retained by
13 Dr. Thompson and by plaintiffs' counsel in this case;
14 correct?
15   MS. O'DELL: Object to the form.
16   THE WITNESS: Yes.
17 BY MR. ZELLERS:
18   Q. As part of that discussion, you were asked
19 and talked about risk factors for ovarian cancer.
20   Do you recall that?
21   A. No.
22   Q. Do you recall that, in that interview in
23 2014, July, you only mentioned age, family history of
24 breast or ovarian cancer, and BRCA genetic mutations
25 as risk factors?

Page 104

1   A. I don't recall that, but it may be on the
2 videotape that you probably have.
3   Q. You did not tell the viewers that talcum
4 powder was associated with or a cause of ovarian
5 cancer; is that right?
6   A. That's correct, because at that point in time
7 I didn't believe it was causative.
8   Q. It wasn't until after being retained in this
9 case, and around the time that you concluded your
10 review in November of 2018, that you formed that
11 opinion; correct?
12   MS. O'DELL: Object to the form.
13   Excuse me. Go ahead.
14   THE WITNESS: As I was preparing to
15 offer an opinion, I did this review and came to that
16 opinion, yes.
17 BY MR. ZELLERS:
18   Q. If we try to put a time on it, it would be
19 toward the latter part of 2018, once you had completed
20 your review that you've told us about in connection
21 with this litigation; correct?
22   A. Yes.
23   MS. O'DELL: Object to the form.
24 BY MR. ZELLERS:
25   Q. Where do practicing gynecological oncologists

Page 105

1 look for guidance on what the risk factors are for
2 ovarian cancer?
3   A. I think a variety of sources, from --
4 published in many textbooks, review articles.
5   Q. Well, just as you don't have the time to go
6 and research each and every potential risk factor for
7 ovarian cancer in depth, you rely on certain
8 organizations to do that research for you; right?
9   MS. O'DELL: Object to the form.
10   THE WITNESS: And other researchers,
11 yes.
12 BY MR. ZELLERS:
13   Q. One organization would be the American
14 College of Obstetricians and Gynecologists, or ACOG;
15 is that right?
16   A. Yes.
17   Q. Another organization would be the Society of
18 Gynecologic Oncology, or SGO; is that right?
19   A. Yes.
20   Q. Another would be the National Cancer
21 Institute's physician data queries?
22   A. I probably wouldn't turn to that, but it's
23 information available to the public.
24   Q. That's generally thought to be reliable
25 information; correct?

Daniel L. Clarke-Pearson, M.D.

Page 106

1   MS. O'DELL: Object to the form.
2   THE WITNESS: I'm not quite certain.
3 I'm not familiar with that. Is this a PDQ you're
4 talking about?
5 BY MR. ZELLERS:
6   Q. A PDQ. But you're familiar, certainly, with
7 the National Cancer Institute; right?
8   A. Yes.
9   Q. The National Cancer Institute has funded at
10 least some of the studies that you have been involved
11 in; is that right?
12   A. As basic research and research into ovarian
13 cancer treatment, not necessarily risk factors.
14   Q. Is it a reputable organization, the National
15 Cancer Institute?
16   A. It's an agency that sponsors cancer research,
17 by and large.
18   Q. Is that a "yes"?
19   A. There -- they're reputable in terms of
20 sponsoring cancer research.
21   Q. You're a member of ACOG; is that right?
22   A. Yes, sir.
23   Q. You're a member of SGO; is that right?
24   A. Yes.
25   Q. You were the president of SGO from 2009 to

Page 107

1 2010; is that right?
2   A. Yeah.
3   Q. You've served on a number of committees for
4 both ACOG and SGO; is that right?
5   A. Yes.
6   Q. Do you agree, generally, that the doctors and
7 scientists in organizations like ACOG and SGO are
8 working very hard to protect women's health?
9   A. Yes.
10   MS. O'DELL: Object to the form.
11 BY MR. ZELLERS:
12   Q. And, in forming your opinions in this case,
13 did you consider the risk factors that ACOG and SGO
14 recognized for ovarian cancer?
15   A. I was familiar with the existing risk factors
16 that had been identified.
17   Q. Are you aware that, even as of today, in
18 their patient-facing websites as well as in their
19 publicly available information about ovarian cancer,
20 neither ACOG nor SGO identify perineal use of talcum
21 powder as a risk factor for ovarian cancer?
22   A. Again, I'm getting back to my point that
23 we're at a point in time where it's a tipping point.
24 And so, yes, right now, that's not posted. And
25 I would imagine that my opinion that ovarian cancer is

Page 108

1 caused by talcum powder will be reflected in those
2 statements in the future.
3   Q. You don't have any reason to believe that the
4 physicians at ACOG and SGO have not kept up to date
5 with the talc and ovarian cancer epidemiology, do you?
6   MS. O'DELL: Object to the form.
7   THE WITNESS: I think that they haven't
8 looked at this question as in depth as I have.
9 BY MR. ZELLERS:
10   Q. How do you know that?
11   A. I'm quite certain of that.
12   Q. Well --
13   A. This is a huge amount of work, to spend 80
14 hours reviewing materials to come to my opinion. I'm
15 not aware of any other physician that's been tasked
16 with that job, if you will.
17   Q. Are there not committees on both ACOG and SGO
18 that look into risk factors and potential causes for
19 ovarian cancer?
20   A. I have served as the committee chair for the
21 GYN Management Committee at ACOG, which publishes
22 committee opinions. And I've also served on the
23 practice committee, which puts out technical
24 bulletins, now called practice bulletins.
25   In both cases, ACOG is asked by a member to

Page 109

1 consider investigating and writing an opinion about
2 that. So if the opinion was requested by an ACOG
3 member, that committee would then decide whether they
4 wanted to pursue that or not.
5   Q. Does ACOG and SGO have committees who
6 generally look at the risk factors for ovarian cancer?
7   A. Only if that committee is asked to look at
8 that question.
9   Q. Any member of ACOG or any member of SGO can
10 ask either ACOG or SGO and their respective committees
11 to look at and evaluate a particular risk factor;
12 correct?
13   A. Yes. Sure.
14   Q. And it's your testimony that that's never
15 ever been done up until today?
16   MS. O'DELL: Object to the form.
17   THE WITNESS: No, it's not my
18 testimony. I don't know what's been requested of ACOG
19 in the past or currently.
20 BY MR. ZELLERS:
21   Q. Would it be important to you to know that
22 Centers for Disease Control and Prevention, the CDC,
23 does not list talcum powder or talc as a risk factor
24 for ovarian cancer?
25   A. That doesn't surprise me.

Daniel L. Clarke-Pearson, M.D.

Page 110

1   Q. The same for the Mayo Clinic. The Mayo
2 Clinic does not list talc as a risk factor for ovarian
3 cancer; correct?
4   A. I'll take your word for it.
5   Q. Have you received funding from the National
6 Institutes of Health?
7   A. I've received funding from the National
8 Cancer Institute, and I have received funding for
9 physician training through the National Institutes of
10 Health for a women's reproductive health research
11 grant.
12   Q. Are you aware that NIH does not list talc as
13 a risk factor for ovarian cancer?
14   A. I would have to look at their publications.
15 That wouldn't surprise me, along with all the other
16 agencies and foundations and organizations that you've
17 listed previously.
18   Q. With respect to the National Cancer
19 Institute, they do publish guidance for physicians on
20 risk factors for cancer; is that right?
21   A. I believe so.
22   Q. Take a look at Deposition Exhibit 18.
23   (Exhibit No. 18 was marked for identification.)
24 BY MR. ZELLERS:
25   Q. Are you familiar with this publication of the

Page 111

1 National Cancer Institute?
2   A. No.
3   Q. This is not something that you reviewed in
4 all of your preparation and research for rendering
5 your opinions in this case?
6   A. I may have seen it, but I'm not familiar with
7 all the details of it.
8   Q. Well, did you review and rely on this
9 statement by the National Cancer Institute with regard
10 to ovarian, fallopian tube, and primary peritoneal
11 cancer prevention in your review of this matter?
12   MS. O'DELL: Object to the form.
13   THE WITNESS: It did not contribute to
14 my formation of my opinion, if that's what you're
15 asking.
16 BY MR. ZELLERS:
17   Q. Well, take a look, if you will, on page 12,
18 12 of 18, at the section "Perineal Talc Exposure."
19   Do you see that?
20   A. Yes.
21   Q. The National Cancer Institute states
22 (as read):
23   "The weight of evidence does not
24   support an association between
25   perineal talc exposure and an

Page 112

1   increased risk of ovarian cancer."
2   Is that right?
3   A. That's what they say.
4   Q. If you go to 18 of 18, this statement was
5 updated as of January 4th of 2019; is that right?
6   MS. O'DELL: Object to the form.
7   THE WITNESS: Yes, I see they updated
8 that.
9   And I think that I do recall having seen
10 this. And my recollection is that their references
11 are not fully up to date too. And also, it befuddles
12 me that the National Cancer Institute -- is that
13 right? -- National Cancer Institute, going back to
14 page 12, would take statistically significant clinical
15 studies and dismiss that clinical significance -- a
16 relative risk of 1.44, a relative risk of 1.26 -- I'm
17 sorry -- 1.71, a relative risk of 1.2 -- and say that
18 they're not important.
19 BY MR. ZELLERS:
20   Q. You have no personal knowledge of the
21 analysis done by the National Cancer Institute that
22 underlie this statement; correct?
23   A. I don't, and I have a hard time understanding
24 how they came to the conclusions they have.
25   Q. Well, let's look at the FDA. The FDA has

Page 113

1 also looked at this issue, has looked at the Bradford
2 Hill factors, and has concluded that causation has not
3 been established as between talcum powder use --
4 peritoneal -- perineal talcum powder use and ovarian
5 cancer; is that right?
6   MS. O'DELL: Object to the form.
7   THE WITNESS: I'd have to see the
8 publication.
9 BY MR. ZELLERS:
10   Q. Well, let's take a look.
11   I'm handing you what we have marked as
12 Deposition Exhibit 19.
13   (Exhibit No. 19 was marked for identification.)
14 BY MR. ZELLERS:
15   Q. This is a letter from the FDA. It has a date
16 stamp at the top, April 1, 2014. It's addressed to
17 Dr. Epstein at the University of Illinois in Chicago.
18   A. I think I have seen this one.
19   Q. FDA is another governmental entity; is that
20 right?
21   A. Yes.
22   Q. As far as you know, the FDA is not biased one
23 way or the other with respect to the food and drug
24 issues that they research and opine on; is that right?
25   MS. O'DELL: Object to the form.

Daniel L. Clarke-Pearson, M.D.

Page 114

1   THE WITNESS: No, that's incorrect. In
2 my personal experience, the FDA has done a bad job in
3 evaluating the risk of morcellation of uterine
4 fibroids. The data that they based their black box
5 opinion on in November of 2014 was based on inadequate
6 review of the medical literature. And it was biased
7 and I think clearly influenced by some outside
8 sources.
9 BY MR. ZELLERS:
10   Q. Do you have criticisms of the FDA's review
11 and investigation of talcum powder products?
12   A. I would like to reread this, because I did
13 have some criticism in reading this.
14   Q. Well, my question is more general. But you
15 would agree --
16   A. Yes, I have criticism. I think that they're
17 not sufficiently evaluating all the data and evidence
18 that's here.
19   Q. Does the FDA have qualified scientists and
20 medical professionals that look at various issues,
21 including talcum powder?
22   MS. O'DELL: Object to the form.
23   THE WITNESS: They probably have
24 qualified people that sometimes make mistakes or
25 sometimes have biases of their own.

Page 115

1 BY MR. ZELLERS:
2   Q. But do you agree that, on scientific issues,
3 including the one that we're here to talk about today,
4 whether or not talcum powder -- genital use of talcum
5 powder is a risk factor for ovarian cancer, that's a
6 topic on which well-qualified scientists and
7 physicians may have differing views?
8   MS. O'DELL: Object to the form.
9   THE WITNESS: They may have differing
10 views, yes.
11 BY MR. ZELLERS:
12   Q. Let's look at this publication from the FDA.
13 Turn to page 4, if you will. And we are looking at
14 Deposition Exhibit 21. Are you at page 4?
15   MS. O'DELL: Are we at 21 or 19?
16   MR. ZELLERS: Oh, I'm sorry.
17 I misspoke. Thank you, Ms. O'Dell. Yes. So let me
18 ask that question again.
19 BY MR. ZELLERS:
20   Q. Turn, if you will, Doctor, to page 4 of
21 Deposition Exhibit 19.
22   THE WITNESS: Ms. O'Dell, may I have --
23 I have some notes on this letter.
24   MS. O'DELL: Is it in your --
25   THE WITNESS: No, I don't think it's in

Page 116

1 the pile.
2 BY MR. ZELLERS:
3   Q. You have notes that are other than what you
4 brought here today?
5   MS. O'DELL: I think it's in -- may be
6 in your stack, Doctor. I'm not sure. I don't have
7 it --
8   THE WITNESS: Well, I'll go through it.
9 My recall of this is this letter is all over
10 the place in terms of pros and cons and pros and cons.
11 So we can work my way through it, but -- go ahead.
12 I'm on page 4.
13 BY MR. ZELLERS:
14   Q. All right. The FDA goes through and reviews
15 epidemiology and etiology findings; is that right?
16   A. That's where they start, yes.
17   Q. The FDA noted, in reviewing this issue,
18 genital use of talcum powder and ovarian cancer, that
19 "selection bias and/or uncontrolled confounding result
20 in spurious positive associations" --
21   A. I'm sorry. Can you just take me to where you
22 are on page 4?
23   Q. Sure. Let's look -- if we're on page 4,
24 right above the findings or conclusion, it says
25 (as read):

Page 117

1   "After consideration of the" --
2   A. My page 4 doesn't have findings and
3 conclusions. "Epidemiology and etiology findings"?
4   Q. Yes. So we're on the same page --
5   A. Above this (indicating)?
6   Q. Underneath "epidemiology and etiology
7 findings" --
8   A. Okay.
9   Q. -- if we go to the second paragraph, it
10 states (as read):
11   "After consideration of the
12   scientific literature submitted in
13   support of both citizen petitions,
14   FDA found..."
15   Are you with me?
16   A. Yes, I am.
17   Q. All right. No. 2 (as read):
18   "The FDA noted that no single
19   study has considered all the
20   factors that potentially
21   contribute to ovarian cancer,
22   including selection bias and/or
23   uncontrolled confounding that
24   result in spurious positive
25   associations between talc use and

Daniel L. Clarke-Pearson, M.D.

Page 118

1   ovarian cancer."
2   Did I read that correctly?
3   A. Yes.
4   Q. You would agree that there are limitations on
5 case-control studies; is that right?
6   A. Yes, there are.
7   Q. There are difficulties in interpreting a
8 retrospective case-control study; is that right?
9   MS. O'DELL: Object to the form.
10   THE WITNESS: I'm not sure what you
11 mean by "difficulties."
12 BY MR. ZELLERS:
13   Q. Well, are there limitations in interpreting a
14 retrospective case-control study?
15   A. There can be.
16   Q. What are those limitations that you're aware
17 of based upon your experience?
18   A. Well, it depends upon how the study is
19 designed, in terms of the size of the study, the --
20 how the -- you know, recall issue is always an issue
21 when you're dealing with patients retrospectively.
22   There are similar problems in cohort studies
23 as well.
24   Q. My question is very simple.
25   What are you aware of in terms of

Page 119

1 limitations of retrospective case-control studies?
2   MS. O'DELL: Object to the form. Asked
3 and answered.
4 BY MR. ZELLERS:
5   Q. That generally apply to case-control studies.
6   MS. O'DELL: Object to the form. Asked
7 and answered.
8   THE WITNESS: Well, there are
9 limitations in probably -- there's a variety of
10 limitations, depending upon the particular studies.
11 So I think we would have to get down to a particular
12 study. And I don't hang my weight -- or hang my hat
13 or put the weight of my opinion on a single study.
14 BY MR. ZELLERS:
15   Q. Well, you would agree that selection bias is
16 a potential concern in case-control studies; correct?
17   A. It can be.
18   Q. And uncontrolled confounding is a potential
19 concern in case-control studies; is that right?
20   A. Yes. But if your controls are well selected,
21 then that negates much of the bias.
22   Q. And, at least in this document, the FDA
23 states that "those result in spurious positive
24 associations between talc use and ovarian cancer
25 risk"; is that right?

Page 120

1   A. That's with regard -- in the first part of
2 their sentence to "no single study."
3   Q. Let's look at Conclusion 3.
4   "The FDA concludes that results of
5   case-control studies do not
6   demonstrate a consistent positive
7   association across studies."
8   Is that right?
9   MS. O'DELL: Objection.
10   THE WITNESS: That's wrong. You read
11 it right; it's wrong.
12 BY MR. ZELLERS:
13   Q. You disagree with the FDA's conclusion; is
14 that right?
15   A. Yes.
16   Q. And I'm going to ask you all about that
17 today --
18   A. Okay.
19   Q. -- so you'll have to chance to tell me why
20 you disagree.
21   Did the FDA also state that, at least based
22 upon its review of the epidemiology and etiology
23 findings, that a dose response -- strike that -- that
24 dose response evidence is lacking?
25   MS. O'DELL: Object to the form.

Page 121

1   THE WITNESS: And can you show me where
2 you're reading that?
3 BY MR. ZELLERS:
4   Q. Sure. Conclusion 3, last part of the
5 statement.
6   A. There is dose response evidence. It's not in
7 every single study, but we are aware of dose
8 response --
9   Q. Doctor, my question was, was it the FDA's
10 conclusion, based upon the epidemiology that it
11 reviewed as of 2014, that dose response evidence is
12 lacking?
13   A. That's the FDA's opinion; that's not my
14 opinion.
15   Q. Finally, the FDA found that "a cogent
16 biological mechanism was lacking." And I'm looking at
17 number 4, "A cogent biological mechanism by which talc
18 might lead to ovarian cancer is lacking."
19   Is that the statement of the FDA, at least
20 as of 2014?
21   A. The statement goes on in the same sentence to
22 say (as read):
23   "Exposure to talc does not account
24   for all cases of ovarian cancer."
25   Nothing accounts for all cases of ovarian

Daniel L. Clarke-Pearson, M.D.

Page 122

1 cancer. I can't believe the FDA would even say

2 something like this.

3   Q. Are you able to answer my question without

4 editorializing?

5   A. I answered your question. I have to finish

6 the whole sentence that you want me to read.

7   Q. Did the FDA state, as of 2014, that "a cogent

8 biological mechanism by which talc might lead to

9 ovarian cancer is lacking"?

10   MS. O'DELL: Object to the form. Asked

11 and answered.

12   THE WITNESS: That's what half of the

13 sentence says. That's what the FDA wrote.

14 BY MR. ZELLERS:

15   Q. All right. IARC, you're certainly familiar

16 with IARC. You brought your whole monograph here with

17 you today; is that right?

18   A. Yes.

19   MS. O'DELL: Object to the form. It's

20 not his monograph; it's not the whole monograph --

21 it's multiple monographs, as you know. So don't --

22 don't be --

23   MR. ZELLERS: I haven't gone through it

24 page by page, but it looks like it's about a

25 2-inch-thick monograph that he brought with him today.

Page 123

1 BY MR. ZELLERS:

2   Q. My question is, are you familiar with IARC?

3   A. I am.

4   Q. All right. IARC has addressed Bradford Hill

5 considerations with respect to talc used in a perineal

6 manner with respect to women -- is that right? -- in

7 ovarian cancer?

8   MS. O'DELL: Object to the form.

9   THE WITNESS: You're asking me a

10 question, not what the FDA is writing here now but

11 what IARC has said?

12 BY MR. ZELLERS:

13   Q. I'm now on to IARC. So let me ask my

14 question.

15   Based upon your review of the IARC

16 monographs, it has addressed the Bradford Hill

17 considerations; is that right?

18   MS. O'DELL: Object to the form.

19   Are you referring to all the monographs?

20 Are you referring to a certain topic that's --

21 because, as you know, there are multiple monographs

22 and they relate to different substances. So, for your

23 specific question, that might be helpful.

24 BY MR. ZELLERS:

25   Q. Are you aware, Dr. Clarke-Pearson, that IARC

Page 124

1 rejected classification of talc as carcinogenic and

2 instead assigned it to the classification of possibly

3 carcinogenic to humans?

4   MS. O'DELL: Object to the form.

5   THE WITNESS: I think that was an IARC

6 publication in the mid 2000s. And I'm aware of it,

7 yes.

8 BY MR. ZELLERS:

9   Q. Are you generally familiar with the IARC

10 categories?

11   A. Generally, but I'm happy to walk through them

12 with you.

13   Q. Sure. Doctor, I show you Exhibit 20.

14   (Exhibit No. 20 was marked for identification.)

15 BY MR. ZELLERS:

16   Q. This is a one-page listing of the agents

17 classified by the IARC monographs, Volumes 1 to 123,

18 and it lists out the different categories that IARC

19 classifies agents within.

20   You're generally familiar with --

21   A. Yes.

22   Q. -- with these classifications; is that right?

23   A. Yes, sir.

24   Q. Looking at Exhibit 20, there are 120 agents

25 in Group 1, "carcinogenic to humans"; is that right?

Page 125

1   A. Yes.

2   Q. That's the only category in which IARC finds

3 sufficient evidence in humans; is that right?

4   A. That's my understanding.

5   Q. And there's 82 agents in Group 2A, "probably

6 carcinogenic to humans"; is that right?

7   A. I see that.

8   Q. It appears that IARC isn't shy about

9 declaring something to be either a known or a probable

10 carcinogen; is that right?

11   MS. O'DELL: Object to the form.

12   THE WITNESS: I don't know about being

13 shy. They have their listing from their --

14 BY MR. ZELLERS:

15   Q. Well, they have over 200 agents in those two

16 categories; is that right?

17   A. Yes.

18   Q. There's only one agent in Group 4, "probably

19 not carcinogenic to humans"; is that right?

20   MS. O'DELL: Object to the form.

21   THE WITNESS: That's what it says.

22 BY MR. ZELLERS:

23   Q. So out of the over a thousand agents that

24 IARC has reviewed, IARC has placed only one agent in

25 Group 4, "probably not carcinogenic"?

Daniel L. Clarke-Pearson, M.D.

Page 126

1   A. Yes.
2   Q. IARC doesn't have a Group 5, "not
3 carcinogenic," do they?
4   A. Not on this sheet.
5   Q. With genital talc, IARC has classified
6 genital talc as a Group 2B category agent; is that
7 right?
8   MS. O'DELL: Object to the form.
9   THE WITNESS: I'm not sure. It's just
10 genital talc. Isn't the talcum powder of all forms?
11 BY MR. ZELLERS:
12   Q. Talcum powder is a Group 2B agent, "possibly
13 carcinogenic to humans"; is that right?
14   A. Yes.
15   Q. That designation is based, according to the
16 IARC definitions, on limited evidence in humans; is
17 that right?
18   MS. O'DELL: Object to the form.
19   THE WITNESS: I would have to read what
20 is written.
21 BY MR. ZELLERS:
22   Q. Is it your understanding that, in classifying
23 talcum powder as a Group 2B agent, that IARC cannot
24 rule out chance, bias, or confounding with reasonable
25 confidence; correct?

Page 127

1   A. I suppose you're reading that from some IARC
2 statement that I don't have, but...
3   Q. That's generally your understanding; correct?
4   A. That would be generally my understanding,
5 yes.
6   Q. Are you aware of some of the other agents
7 that have been designated as 2B agents by IARC as
8 possibly carcinogenic?
9   A. I am not.
10   Q. Ginkgo biloba? Are you familiar with that?
11   A. No.
12   Q. Occupational carpentry and joinery?
13   MS. O'DELL: I'm sorry. I missed that
14 last one. What did you say?
15 BY MR. ZELLERS:
16   Q. Occupational carpentry and joinery.
17   A. I was not aware of that.
18   Q. Pickled vegetables?
19   A. I've heard that.
20   Q. All right. What association does the
21 literature report between talc use and ovarian cancer?
22   A. Well, now we move into looking at
23 epidemiology, in my opinion.
24   Q. Well, these are just a few general questions.
25 If you need to look at your folders, please do. But

Page 128

1 I just have a few general questions.
2   A. All right. Well, please go ahead.
3   Q. Well, are you able to tell me, generally,
4 what association the literature reports between talc
5 use and ovarian cancer?
6   A. The literature consistently shows an
7 increased risk of developing ovarian cancer in women
8 that are exposed to talcum powder.
9   Q. Generally, it's around a 1.3 odds ratio in
10 the case-control studies; is that fair?
11   MS. O'DELL: Object to the form.
12   THE WITNESS: I would acknowledge that,
13 yes.
14 BY MR. ZELLERS:
15   Q. All right. Do you also acknowledge that the
16 epidemiologists consider a 1.3 odds ratio in
17 case-control studies to be a weak or modest
18 association?
19   MS. O'DELL: Object to the form.
20   THE WITNESS: I'm not sure what they
21 mean by "weak" or "modest."
22 BY MR. ZELLERS:
23   Q. Would you categorize it as a weak or modest
24 association?
25   A. No. I would call it a statistically

Page 129

1 significant observation that impacts the lives of
2 thousands of women that I've taken care of over the
3 years and that, if talcum powder were not on the
4 market and being used in perineal hygiene, for lack of
5 a better word, many other women would not have died of
6 ovarian cancer that I've taken care of.
7   MR. ZELLERS: Move to strike as
8 nonresponsive.
9 BY MR. ZELLERS:
10   Q. You are unaware as to whether or not an
11 epidemiologist would consider a 1.3 odds ratio in a
12 case-control study to be a weak or modest association;
13 is that right?
14   A. I don't understand the definition of "weak"
15 or "modest."
16   Q. You're not an epidemiologist; is that right?
17   A. That's correct.
18   Q. Can you point to any peer-reviewed literature
19 on talc and ovarian cancer that states that 1.3 odds
20 ratio is a strong association?
21   A. I think --
22   MS. O'DELL: Object to the form.
23   THE WITNESS: -- it's a statistically
24 significant association that's been consistently
25 reported in case-control studies and in meta-analyses.

Daniel L. Clarke-Pearson, M.D.

Page 130

1 BY MR. ZELLERS:
2   Q. I take it that's no to my question. Is that
3 right? And I'll ask it again if you'd like me to.
4   MS. O'DELL: Object to the form.
5 I think he answered your question.
6   THE WITNESS: I'm not aware that it's a
7 strong association or a weak association. It's a
8 statistically significant association.
9 BY MR. ZELLERS:
10   Q. You cannot point me to any peer-reviewed
11 literature on talc and ovarian cancer that states that
12 1.3 is a strong association; correct?
13   MS. O'DELL: Object to the form. Asked
14 and answered.
15   THE WITNESS: That's correct.
16 BY MR. ZELLERS:
17   Q. IARC does not refer to this as a strong
18 association; correct?
19   A. I'm not familiar with what IARC says.
20   Q. FDA does not refer to this as a strong
21 association; correct?
22   A. I'm not aware.
23   Q. The National Cancer Institute does not refer
24 to this as a strong association; correct?
25   A. I'm not aware what they said about strong or

Page 131

1 weak.
2   Q. Do your opinions on causation and strength of
3 association apply equally to all forms of ovarian
4 cancer?
5   A. No.
6   Q. Are you able to break down your opinion with
7 respect to ovarian cancer?
8   A. Yeah. So there are three types of ovarian
9 cancer: germ cell, sex cord-stromal, and epithelial
10 ovarian cancers. I have no evidence that sex
11 cord-stromal tumors or germ cell tumors are associated
12 with the use of talcum powder, although they are rare
13 cancers, so it would take much larger populations to
14 really fully investigate that issue.
15   Q. Do you -- strike that.
16   Does your opinion on strength of association
17 and causation apply equally to all forms of epithelial
18 ovarian cancer?
19   A. Reading the literature, it appears that there
20 is some variation in terms of impact that talcum
21 powder might have on some forms of ovarian cancer.
22   Q. Tell us what your opinions with the different
23 subtypes of epithelial ovarian cancer and whether or
24 not they are either a risk factor or a causative
25 factor for ovarian cancer.

Page 132

1   MS. O'DELL: Object to the form.
2   THE WITNESS: I'm not sure that
3 question --
4 BY MR. ZELLERS:
5   Q. I thought it was a good question. I can try
6 to do it again, but, did you not understand that
7 question?
8   A. I think what you're trying to get at is does
9 talcum powder have equal carcinogenic effect resulting
10 in different types of epithelial ovarian cancers?
11   Q. Yes.
12   A. Okay. So different types of epithelial
13 ovarian cancers are separated into several -- and we
14 believe there are several different mechanisms that
15 cause them. So in the past, they've been lumped into
16 epithelial ovarian cancers; but, in fact, the biology
17 of mucinous tumors -- cancers -- are different than
18 serous cancers.
19   Based on the epidemiologic evidence that
20 I've seen, there is a preponderance of impact on women
21 that have serous carcinomas of the ovary, which is the
22 most common ovarian cancer; and because it is the most
23 common, it's more likely we're going to see a
24 statistical association as opposed to a rarer cancer
25 like a mucinous cancer.

Page 133

1   So that is my answer to your question.
2   Q. Do your opinions as to talcum powder used in
3 the perineal area being a risk factor and/or a
4 causative factor for serous ovarian cancer also apply
5 to mucinous ovarian cancer?
6   A. I think the association is weaker for
7 mucinous.
8   Q. How about for endometrioid?
9   A. I think some studies have suggested
10 endometrioid is increased risk with talcum powder.
11   Q. Is it weaker?
12   A. Is it weaker?
13   Q. Than serous.
14   A. Than serous? I'm not certain of that.
15   Q. Clear cell, is it weaker than serous?
16   A. I'm not certain of that because clear cell is
17 a very rare cancer.
18   Q. On page 8 of your report, you say that
19 (as read):
20   "The strength of association
21   between talcum powder and ovarian
22   cancer is critically important
23   because of severity and frequency
24   of ovarian cancer."
25   Is that right?

Daniel L. Clarke-Pearson, M.D.

Page 134

1   A. That's what I say.

2   Q. Do you believe that ovarian cancer is a

3 frequently occurring disease?

4   A. In my practice it is. It occurs in 22,400

5 women a year in the United States, and about 14,000 of

6 those women will ultimately die of their cancer.

7   Q. What is your support for that?

8   A. My support for that data, the incidence of

9 ovarian cancer?

10   Q. Yes.

11   A. Well, I may have rounded it off and it may

12 not be exact, but the American -- I mean the American

13 Cancer Society, the SEER database. Those would be two

14 sources of information that count the annual incidence

15 of ovarian cancer and the mortality from ovarian

16 cancer.

17   Q. When you examine a causation, are you more

18 likely to consider a lower association causal if the

19 disease is severe or frequent?

20   MS. O'DELL: Object to the form.

21   THE WITNESS: Let me read your question

22 again.

23   I'm not sure what you mean by "lower

24 association."

25

Page 135

1 BY MR. ZELLERS:

2   Q. You have told us in your report that "the

3 strength of association between talcum powder and

4 ovarian cancer is critically important because of the

5 severity and frequency of ovarian cancer."

6   Is that right?

7   A. Yes, that's right.

8   Q. My question is, when you examine causation,

9 are you more likely to consider a lower association

10 causal if the disease is severe or frequent?

11   MS. O'DELL: Object to the form.

12   THE WITNESS: No, it doesn't have

13 anything to do with my opinion as to what the

14 causation is.

15 BY MR. ZELLERS:

16   Q. Langseth, 2008, that is a study that you have

17 reviewed and that you rely upon for your opinions in

18 this case; is that right?

19   A. I believe so. It's one of the meta-analyses,

20 as I recall.

21   Q. Are you familiar with the Langseth

22 publication?

23   A. I have read it, and I think it's of value,

24 but --

25   Q. Take a look at -- I'm going to hand you the

Page 136

1 exhibit copy.

2   A. Sure.

3   Q. We have marked this one as Exhibit 21.

4   (Exhibit No. 21 was marked for identification.)

5   THE WITNESS: Okay.

6   MS. O'DELL: Feel free to look at your

7 own copy if you'd rather, Doctor.

8 BY MR. ZELLERS:

9   Q. Do you have Exhibit 21?

10   A. Yes. You gave me two copies. Here, let me

11 give you one back.

12   Q. Ah, okay.

13   You have both the exhibit copy I gave you,

14 which is not highlighted, and you have your own

15 personal highlighted copy of the study; is that right?

16   A. Yes, sir.

17   Q. On page 7 of your report, you address this

18 meta-analysis by Langseth; is that right?

19   A. I've lost track of my report, but as soon as

20 I get to it -- here we go.

21   Q. Your report is Exhibit 5; is that right?

22   A. I have one that's not marked, but go ahead.

23   Q. Well, turn to page 7.

24   A. Mm-hmm.

25   Q. And do you see in your chart you have

Page 137

1 identified Langseth as one of the six articles that

2 you have pulled out and highlighted in your paper; is

3 that right?

4   A. Yes.

5   Q. And you list the odds ratio found by Langseth

6 and the other authors in that paper to be 1.40; is

7 that right?

8   A. That's correct.

9   Q. Go to Figure 1 on page 359 of the Langseth

10 article, Exhibit 21.

11   Do you have that?

12   A. Yes.

13   Q. And Langseth lists 20 case-control studies;

14 is that right?

15   A. I believe so.

16   Q. Of those 20 studies, only 10 have

17 statistically significant results; is that right?

18   A. I'm going to have to go through each one, so

19 give me a moment here.

20   I count 11.

21   Q. You count 11 that found a statistical

22 significance?

23   A. Where the confidence interval does not

24 overlap 1.

25   Q. Well, we have Cramer; correct?

Daniel L. Clarke-Pearson, M.D.

Page 138

1   A. Yes.
2   Q. Second, Harlow; correct?
3   A. Yes.
4   Q. Cramer again; correct?
5   A. Yes.
6   Q. Purdie; is that right?
7   A. Yes.
8   Q. Chang?
9   A. Yes.
10   Q. Cook?
11   A. Yes.
12   Q. Green?
13   A. Yep.
14   Q. Cramer?
15   A. Yep.
16   Q. Ness?
17   A. Yes.
18   Q. Mills?
19   A. Yes.
20   Q. That's 10. You see another one?
21   A. Okay. I'm sorry. I counted the pooled odds
22   ratio population-based studies. So 10. Yes, I agree
23   with you.
24   Q. So out of the 20 case-control studies that
25   are cited by Langseth and that you rely on for your

Page 139

1   opinions in this matter, only 10 of the 20 have
2   statistically significant results; is that right?
3   A. Yes.
4   Q. Is this the first time that you've done that
5   exercise, that you've actually looked at the 20
6   studies and determined that only 10 of them have
7   statistically significant results?
8   MS. O'DELL: Object to the form.
9   THE WITNESS: No. I didn't go through
10   every -- to count -- let me read your question again.
11   I was not aware of the exact count that you
12   brought to my attention. On the other hand, I think
13   that this paper results in a statistically significant
14   finding. That's the beauty of a meta-analysis.
15   BY MR. ZELLERS:
16   Q. Would you agree that 10 out of 20 is no
17   better than a coin toss?
18   MS. O'DELL: Object to the form.
19   THE WITNESS: You're misusing
20   epidemiologic data.
21   BY MR. ZELLERS:
22   Q. Would you agree that 10 out of 20 is no
23   better than a coin toss?
24   MS. O'DELL: Object to the form.
25   THE WITNESS: You'll have to tell me

Page 140

1   what 10 out of 20 we're talking about.
2   MS. O'DELL: Sorry, Doctor. Object to
3   the form. Asked and answered.
4   You may answer his question.
5   BY MR. ZELLERS:
6   Q. Generally, if you flip a coin 20 times, are
7   you going to get 10 heads and 10 tails?
8   MS. O'DELL: Object to the form.
9   THE WITNESS: Statistically, yes.
10   BY MR. ZELLERS:
11   Q. All right. Is it your opinion that 10 out of
12   20 means there are consistent results across
13   studies --
14   A. That's where a meta-analysis puts weight onto
15   some studies more than others.
16   Q. The --
17   A. -- and comes up with a conclusion that this
18   is a statistically significant finding, pooling all of
19   these papers.
20   Q. Langseth is just looking at the case-control
21   studies; is that right?
22   A. Yes.
23   Q. Langseth concluded -- and the authors
24   concluded -- that causation should be rejected and
25   that more study is needed; is that right?

Page 141

1   MS. O'DELL: Object to the form.
2   THE WITNESS: I'd have to see where
3   that's written.
4   BY MR. ZELLERS:
5   Q. Well, look under -- so same page, underneath
6   our table, see where it says "Proposal to research
7   community"?
8   A. Yes.
9   Q. (As read):
10   "The current body of experimental
11   and epidemiological evidence is
12   insufficient to establish a causal
13   association between perineal use
14   of talc and ovarian cancer risk."
15   Did I read that correctly?
16   A. You read that correctly.
17   Q. Would you agree that you're drawing
18   conclusions from this study that are broader than the
19   study authors' own conclusions?
20   MS. O'DELL: Object to the form.
21   THE WITNESS: My opinion is not based
22   on just this study; it's based on all of the studies
23   that I have in my report where there's a consistency
24   across all meta-analyses that there's a statistically
25   increased risk of ovarian cancer in women exposed to

Daniel L. Clarke-Pearson, M.D.

Page 142

1 perineal talc. Those confidence intervals in all of
2 those meta-analyses are statistically significant.
3   MR. ZELLERS: Move to strike as
4 nonresponsive.
5 BY MR. ZELLERS
6   Q. Are these -- at least with the Langseth
7 paper, you've gone further than what the authors have
8 concluded; correct?
9   MS. O'DELL: Object to the form.
10   THE WITNESS: I'm developing my opinion
11 on the totality of the evidence that I have reviewed.
12 BY MR. ZELLERS:
13   Q. Please answer my question. Just on the
14 Langseth paper --
15   A. My opinion is not based on the Langseth
16 paper.
17   Q. I understand. But with respect to Langseth
18 and the opinions that you've drawn from Langseth,
19 you've gone further in your conclusions than the
20 Langseth paper authors; correct?
21   A. No, I do not.
22   MS. O'DELL: Excuse me.
23   Object to the form. Misstates his
24 testimony.
25   You may repeat your answer if you'd like.

Page 143

1   THE WITNESS: My conclusions are not
2 based on only Langseth. That is a piece of
3 information that I've used in formulating my opinion.
4 BY MR. ZELLERS:
5   Q. Consistency is one of the Bradford Hill
6 factors; is that right?
7   A. Yes, sir.
8   Q. On page 6 of your report, you discuss the
9 epidemiological studies on talcum powder and ovarian
10 cancer; is that right?
11   A. Yes.
12   Q. In the second paragraph, under
13 "Epidemiology," you state (as read):
14   "When looking at these
15   epidemiologic studies and their
16   totality, the data shows a
17   consistent statistically
18   significant increased risk of
19   developing EOC [epithelial ovarian
20   cancer] with perineal talcum
21   powder use."
22   Is that right?
23   A. Yes, sir.
24   Q. In looking at this section, you don't discuss
25 or address the cohort studies at all; is that right?

Page 144

1   A. That's right.
2   Q. You just discuss the case-control studies and
3 then the meta-analyses; is that right?
4   A. That's correct.
5   MS. O'DELL: Object to the form.
6 BY MR. ZELLERS
7   Q. The cohort studies do not show a
8 statistically significant association between talc use
9 and ovarian cancer; is that right?
10   A. The cohort studies were not designed to
11 answer that question. They're poorly done and I don't
12 think contribute to this discussion.
13   Q. Is that a "yes," that the cohort studies do
14 not show a statistically significant association
15 between talc use and ovarian cancer?
16   A. The way they're written and studied and
17 reported, you're correct.
18   Q. Berge 2017, that's a paper you've got in one
19 of your folders that we went through earlier today.
20 And you're generally familiar with that study; is that
21 right?
22   A. Yes.
23   Q. In Berge, the authors concluded that
24 (as read):
25   "The positive association between

Page 145

1   talc use and ovarian cancer
2   appears to be limited to serous
3   histologic type and to
4   case-control studies."
5   Do you agree with that?
6   A. Yes.
7   Q. How can you validate completely excluding
8 cohort studies from your discussion?
9   MS. O'DELL: Object to the form.
10   THE WITNESS: Because I don't think
11 they contribute one way or the other. They're poorly
12 designed, poorly executed, and the data that they
13 provide does not inform us at all.
14   And, in fact, these meta-analyses, in many
15 cases, included the cohort studies and still came out
16 with statistically significant increased risk of
17 ovarian cancer.
18 BY MR. ZELLERS:
19   Q. It was appropriate for you to exclude the
20 cohort studies from your discussion; correct?
21   MS. O'DELL: Object --
22   THE WITNESS: I did --
23   MS. O'DELL: Excuse me. Object to the
24 form. Misstates his testimony.
25   You may answer.

Daniel L. Clarke-Pearson, M.D.

Page 146

1   THE WITNESS: This table back here
2 that's got all these papers on it, we excluded.
3 They're not in my discussion. I considered them, and
4 I didn't think that they contributed to the
5 information that I needed to present in my report.
6 BY MR. ZELLERS:
7   Q. You state that Penninkilampi shows that the
8 cohort studies support a statistically -- well, strike
9 that.
10   I want to ask you a few questions about the
11 cohort studies.
12   Did you review the Gates 2010 cohort study?
13   A. Yes.
14   Q. The Gates 2010 cohort study found that there
15 was not a statistically significant relationship for
16 the serous invasive subtype of ovarian cancer; is that
17 right?
18   A. I believe that's true, from my recollection.
19   Q. Berge 2017 shows that the cohort studies do
20 not support a statistically significant relationship
21 between perineal talc use and ovarian cancer for any
22 subtype; is that right?
23   MS. O'DELL: Object to the form.
24   THE WITNESS: This is Berge's analysis
25 of the cohort studies and Berge's meta-analysis. Is

Page 147

1 that the paper you're talking about?
2 BY MR. ZELLERS:
3   Q. Yes. 2017.
4   A. I presume, if you're reading it, that's what
5 he says.
6   Q. Well, I'm looking at Berge 2017, page 6, left
7 column, at the bottom (as read):
8   "This positive association appears
9   to be limited to serous histologic
10   type and the case-control
11   studies."
12   We covered that earlier; correct?
13   A. Yes.
14   MS. O'DELL: What page, please?
15   MR. ZELLERS: Page 6.
16 BY MR. ZELLERS:
17   Q. We're in agreement on that; correct, Doctor?
18   MS. O'DELL: Object to the form. Give
19 him a moment.
20   THE WITNESS: Yes, he says that in his
21 abstract.
22 BY MR. ZELLERS:
23   Q. You were aware that Berge 2017 included the
24 Gates 2010 cohort study; is that right?
25   A. Yes. It's in Figure 2.

Page 148

1   Q. You're aware that one of the studies --
2 another one of the meta-analyses that you cite to,
3 Penninkilampi 2018, excludes the Gates 2010 cohort
4 study; right?
5   A. I believe so.
6   Q. How did you make a determination to weigh
7 Penninkilampi more heavily than Berge?
8   They're both meta-analyses; correct?
9   A. Right.
10   Q. Why did you make a determination to weigh
11 Penninkilampi 2018 and place greater weight on it than
12 the Berge study?
13   MS. O'DELL: Object to the form.
14   THE WITNESS: I don't think
15 I necessarily placed greater weight on it. I've told
16 you how I weight studies, and they all contribute to
17 the totality of my opinion.
18 BY MR. ZELLERS:
19   Q. Did you -- well, strike that.
20   Isn't it a problem that Penninkilampi 2018
21 does not factor in the data from the Gates 2010 study,
22 given that the Gates study tends to negate an
23 association between perineal talc use and ovarian
24 cancer?
25   MS. O'DELL: Object to the form.

Page 149

1   THE WITNESS: I can't explain to you
2 what Penninkilampi was thinking or why he chose to
3 exclude it.
4 BY MR. ZELLERS:
5   Q. Did you verify that the data that
6 Penninkilampi reports is accurate?
7   A. Have I gone through every single case-control
8 study and verified every number that's in his tables?
9   Q. Have you -- strike that.
10   Penninkilampi purports to report odds
11 ratios, lower limits and upper limits, for the
12 individual studies; is that right?
13   A. Yes.
14   Q. Did you go back to verify that Penninkilampi
15 was correct in his reporting of the results of those
16 individual studies?
17   A. Yeah, that's the question I was just asking
18 you.
19   No, I did not go back.
20   Q. In determining the study is of high quality,
21 would it be important to you that the authors are
22 accurately reporting the odds ratios and the
23 confidence intervals?
24   MS. O'DELL: Object to the form.
25   THE WITNESS: I trust the peer review

Daniel L. Clarke-Pearson, M.D.

Page 150

1 process that resulted in this publication.
2 BY MR. ZELLERS:
3   Q. If there were errors in reporting of the odds
4 ratios or the confidence intervals, would that call
5 into question the reliability of the study?
6   MS. O'DELL: Object to the form.
7   THE WITNESS: It might.
8 BY MR. ZELLERS:
9   Q. Of the histological subtypes for epithelial
10 ovarian cancer, do you consider endometrioid and clear
11 cell to be related?
12   A. No.
13   Q. You do not consider endometrioid and clear
14 cell ovarian cancer to be related?
15   A. Only related in they fall into the
16 classification of epithelial ovarian cancers.
17   Q. Penninkilampi only found a statistically
18 significant increased risk for serous and endometrioid
19 ovarian cancers; is that right?
20   A. Okay. Yes.
21   MS. O'DELL: Let -- excuse me, Doctor.
22   If you need to look at the --
23 BY MR. ZELLERS:
24   Q. You have Penninkilampi in front of you,
25 right, Doctor?

Page 151

1   A. I have.
2   Q. And if you need to take any more time to
3 answer any of my questions, please do.
4   A. Okay.
5   Q. Penninkilampi did not find a statistically
6 significant increased risk for clear cell or mucinous
7 ovarian cancer; is that right?
8   A. Can you show me where you're reading it from?
9   Q. Sure. Take a look at the abstract for the
10 results.
11   A. He says he found an increased risk of serous
12 and endometrioid but not mucinous or clear cell.
13   Q. And that's where I was going to. So our
14 record is complete, let's mark -- well, let's mark
15 both Berge 2017 -- we'll mark Berge 2017.
16   MS. O'DELL: Mike, I think there's an
17 updated Berge publication, 2018. Do you have the most
18 up to date?
19   MR. ZELLERS: Asking him a question
20 about the Berge publication copyrighted 2017 that
21 appeared in "Genital Use of Talc and Risk of Ovarian
22 Cancer, a Meta-analysis." That's the one that I'm
23 referring to and I believe the one that the doctor has
24 identified in his materials.
25   THE WITNESS: Actually, mine is from

Page 152

1 May of 2018, European Journal of Cancer Prevention.
2 BY MR. ZELLERS:
3   Q. Okay. So let's do this: Doctor, if you
4 don't mind, hand me your copy. We'll mark that as
5 Deposition Exhibit 23.
6   MR. ZELLERS: For right now, I'm going
7 to just put a No. 23. And, Ms. Court Reporter, if, at
8 a break, you can put an official sticker on it.
9   MS. O'DELL: I hate to even say this,
10 but did we mark 22?
11   MR. ZELLERS: Yes. So Deposition
12 Exhibit 22 is the Berge 2017 paper.
13   Deposition Exhibit 23 is the Berge
14 publication that appeared in the European Journal of
15 Cancer Prevention, dated May 2018.
16   (Exhibit Nos. 22 and 23 were marked for
17   identification.)
18 BY MR. ZELLERS:
19   Q. So I'm going to hand both of these back to
20 you, Dr. Clarke-Pearson.
21   MR. ZELLERS: I'm going to hand out my
22 exhibit copies to counsel.
23   Let me also, just so we have it in the
24 record, we'll mark as Deposition Exhibit 24 the
25 Penninkilampi meta-analysis that's referred to in the

Page 153

1 doctor's report.
2   (Exhibit No. 24 was marked for identification.)
3 BY MR. ZELLERS:
4   Q. All right, Doctor. Can I ask you some more
5 questions?
6   A. Let's go for it.
7   Q. Does it make sense that an environmental
8 exposure could increase the risk for endometrioid
9 ovarian cancer but not clear cell ovarian cancer?
10   MS. O'DELL: Object to the form.
11   THE WITNESS: Yes.
12 BY MR. ZELLERS:
13   Q. How do you explain that finding?
14   A. Well, we've talked about mutations
15 previously, and I'll bring it up again, that different
16 mutations occur that result in different types of
17 cancers. And so the ovarian epithelium being exposed
18 to talcum powder may develop different cancers,
19 depending upon the impact that that talcum powder and
20 its products have on that particular cell.
21   Q. Do you believe -- and, I think, as you told
22 us earlier -- that you find a stronger association
23 between perineal talcum powder use and serous ovarian
24 cancer than you find for endometrioid, clear cell, or
25 mucinous ovarian cancer; is that right?

Daniel L. Clarke-Pearson, M.D.

Page 154

1   MS. O'DELL: Object to the form.
2   THE WITNESS: I think serous has the
3 strongest association. But in some studies we see,
4 just as you're quoting from the -- whichever the study
5 is that we're looking at, that endometrioid -- the
6 Penninkilampi study -- so serous and endometrioid is
7 increased.
8 BY MR. ZELLERS:
9   Q. But not clear cell or mucinous; correct?
10   A. That's correct in this one study.
11   Q. Do you believe that Penninkilampi 2018
12 provides evidence that there's a biologically
13 plausible mechanism by which talc can cause ovarian
14 cancer?
15   A. I don't recall, and I'm not seeing it as I do
16 a quick scan, that he addresses mechanisms of
17 cancer -- carcinogenesis. I wouldn't expect that in
18 an epidemiologic study.
19   Q. Penninkilampi specifically states that
20 (as read):
21   "A certain causal link between
22   talc use and ovarian cancer has
23   not been established."
24   Correct?
25   MS. O'DELL: Object to the form.

Page 155

1   THE WITNESS: That's what he has
2 written, and you've read it correctly.
3   MS. O'DELL: Are you reading at a
4 certain page, Counsel?
5   MR. ZELLERS: Yes. I was reading from
6 page 42, the end of the first paragraph.
7   THE WITNESS: Okay. Right.
8 BY MR. ZELLERS:
9   Q. Did I read that correctly? It's the last
10 statement in the first paragraph in the left-hand side
11 (as read):
12   "A certain causal link between
13   talc use and ovarian cancer has
14   not yet been established."
15   Did I read that correctly?
16   A. I'm sorry. I'm losing track of where you
17 are. Are you up here?
18   Q. Right here (indicating).
19   A. Okay. Yes, you read it correctly.
20   Q. Cohort studies are not affected by recall
21 bias; is that right?
22   A. Not by recall bias, no.
23   Q. All of the cohort studies were prospective as
24 opposed to retrospective; is that right?
25   A. The cohort studies gathered information about

Page 156

1 exposure at one point in time and never followed the
2 patients subsequent to that to get some idea of
3 frequency of use, whether the patient continued to use
4 the talcum powder so that the real question is ever
5 use. We don't know duration and frequency from these
6 cohort.
7   MR. ZELLERS: Move to strike as
8 nonresponsive.
9   MS. O'DELL: Oppose the motion.
10   MR. ZELLERS: And, Counsel,
11 I understand that anytime I do that, you will oppose
12 it.
13   MS. O'DELL: I just wanted to make it
14 clear. Didn't want you to think I was asleep over
15 here.
16   MR. ZELLERS: I'm going to ask my
17 question again.
18 BY MR. ZELLERS:
19   Q. Dr. Clarke-Pearson, all of the cohort studies
20 were prospective as opposed to retrospective; correct?
21   A. They're prospective except for the fact that
22 they don't continue to evaluate the ongoing use of
23 talc in these patients. It was a point in time that
24 the patient was asked whether she did or didn't use
25 talc.

Page 157

1   Q. The cohort studies were not subject to the
2 same selection bias as retrospective case-control
3 studies; is that right?
4   A. That's true.
5   Q. Recall bias is a concern in every
6 retrospective study; correct?
7   A. Yes.
8   Q. Recall bias can distort a scientific
9 evaluation of whether an exposure is actually related
10 to a disease; correct?
11   MS. O'DELL: Object to the form.
12   THE WITNESS: Let me read your question
13 again.
14   Recall bias has that risk of not being able
15 to analyze the data.
16 BY MR. ZELLERS:
17   Q. For example, recall bias could distort
18 results if women with ovarian cancer were more likely
19 to remember their exposure to talc than women without
20 ovarian cancer; is that right?
21   MS. O'DELL: Object to the form.
22   THE WITNESS: The issue in these large
23 case-control trials is that we have many, many more
24 women in them that have ovarian cancer. And,
25 therefore, those potentially confounding factors get

Daniel L. Clarke-Pearson, M.D.

Page 158

1 worked out in most cases, and there is a consistency
2 across all of these studies.
3 BY MR. ZELLERS:
4   Q. I'm going to ask you about consistency. I'm
5 going to ask you about confounding factors. But, for
6 right now, please try to answer my question.
7   Recall bias could distort results if women
8 with ovarian cancer were more likely to remember their
9 exposure to talc than women without ovarian cancer;
10 correct?
11   A. Yes, that could distort the results.
12   Q. Recall bias could explain the fact that some
13 retrospective case-control studies have found a
14 statistically significant relationship between talcum
15 powder and ovarian cancer but the cohort studies have
16 not; correct?
17   MS. O'DELL: Object to the form.
18   THE WITNESS: (As read):
19   "Recall bias could explain the
20   fact that some retrospective
21   case-control studies have found a
22   statistically significant
23   relationship between talcum powder
24   and ovarian cancer?"
25   Yes, that's true.

Page 159

1   And then you go on to say "but the cohort
2 studies have not."
3   Have not found a statistically significant
4 relationship? That's true. The cohort studies
5 haven't found a statistically -- because the cohort
6 studies have many other confounding and inadequate
7 parts of their evaluation.
8   MR. ZELLERS: Move to strike as
9 nonresponsive.
10 BY MR. ZELLERS:
11   Q. You rely on the Schildkraut case-control 2016
12 study for your opinions about dose response; is that
13 right?
14   A. About what response?
15   Q. About dose response.
16   A. Dose response? That's one of the studies.
17   Q. All right. Take a look, if you will, please,
18 at Deposition Exhibit 25, which is the Schildkraut
19 2016 study cited and relied upon by you.
20   (Exhibit No. 25 was marked for identification.)
21 BY MR. ZELLERS:
22   Q. Do you have that in front of you?
23   A. Yes. You just handed it to me.
24   Q. And this is a study that you have previously
25 reviewed and you cite to in your materials in this

Page 160

1 case; is that right?
2   A. Yes.
3   Q. Schildkraut 2016 looked at, among other
4 things, what impact, if any, lawsuit filings in 2014
5 had had on whether women recalled using talc in the
6 past; is that right?
7   A. I think she tried to evaluate that, yes.
8   Q. The authors thought that the publicity from
9 the lawsuits might influence the participants' recall
10 of prior body powder use; is that right?
11   A. Yes.
12   Q. If we go to page 4 of Exhibit 25 --
13   A. Page 1414, Table 2?
14   Q. Yeah. Page 1414, Table 2, the second column
15 shows the number of cases. That's women with ovarian
16 cancer; is that right?
17   A. Yes.
18   Q. The third column shows the controls. Those
19 are the women who do not have ovarian cancer; is that
20 right?
21   A. That's correct.
22   Q. Looking at this data, before 2014, before the
23 lawsuits, the percentage of controls -- meaning women
24 without ovarian cancer -- who said they used talc on
25 their genitals was 34 percent; is that right?

Page 161

1   A. That's not in this table, I don't think, is
2 it?
3   Q. Take a look -- do you see, under "Exposure,"
4 "Body powder use by location"? It's about eight lines
5 down, "Interview date, less than or earlier than
6 2014."
7   A. I'm with you, yeah. Okay.
8   Q. All right. So the percentage of controls --
9 meaning women without ovarian cancer -- who said they
10 used talc on their genitals was 34 percent; is that
11 right?
12   A. I'm not seeing that. I see "interview date
13 less than 2014, never used."
14   Q. Then you go down to "any genital use."
15   A. Okay. "Any genital use, 34 percent," yes.
16 I see what you're saying.
17   Q. And then the percentage of cases -- meaning
18 women with ovarian cancer -- that they said used talc
19 on their genitals who were interviewed before 2014 was
20 36.5 percent; is that right?
21   A. Right. That's correct.
22   Q. So roughly the same reporting of genital talc
23 use between women with and without ovarian cancer
24 before the lawsuits were filed; is that right?
25   A. Yes.

Daniel L. Clarke-Pearson, M.D.

Page 162

1   Q. Now, look at what happened after the lawsuits
2 were filed.
3   A. I see.
4   Q. After 2014, what percent of women without
5 ovarian cancer said they used talc on their genitals?
6   A. 34.4 percent.
7   Q. So essentially the same as before; is that
8 right?
9   A. Yes.
10   Q. So, based on this data, the lawsuits had
11 essentially no effect on how many of the women without
12 ovarian cancer, the controls, remembered or recalled
13 using baby powder; is that right?
14   A. That seems to be true.
15   Q. For women with ovarian cancer, as we
16 discussed, before the lawsuits were filed,
17 36.5 percent of them said they recalled using baby
18 powder; is that right?
19   A. Yes.
20   Q. But after the lawsuits were filed,
21 the percent of women with ovarian cancer who said they
22 used baby powder went up to 51.5 percent; is that
23 right?
24   A. That's correct.
25   Q. So after the lawsuits were filed, the percent

Page 163

1 of women with ovarian cancer who said they used baby
2 powder jumped by over 40 percent; is that right?
3   A. It went from 36.5 to 51.5.
4   Q. That's just over 40 percent; correct? That
5 increase?
6   A. From 36 to 51?
7   Q. Yes.
8   A. You're doing the math, but --
9   Q. Well, it's a substantial increase.
10   A. Yes.
11   Q. Would you agree with that?
12   MS. O'DELL: Object to the form.
13   THE WITNESS: Yes.
14 BY MR. ZELLERS:
15   Q. All right. So, looking at this data, lawsuit
16 filings affected how many women with ovarian cancer
17 remembered using talc on their genitals but basically
18 had no effect on the memory of women without ovarian
19 cancer; correct?
20   MS. O'DELL: Object to the form.
21   THE WITNESS: I don't know that it --
22 the hypothesis that Dr. Schildkraut puts out there is
23 that the lawsuit filings may have changed women's
24 recall, if you will. There may be other factors that
25 are involved here too.

Page 164

1 BY MR. ZELLERS:
2   Q. At least according to the author, the women,
3 after a lawsuit was filed, with ovarian cancer
4 remembered using talc much more than the women without
5 ovarian cancer; correct?
6   A. Yes.
7   MS. O'DELL: Object to the form.
8 BY MR. ZELLERS:
9   Q. Those findings would be an example of the
10 potential effect of recall bias; is that right?
11   A. Yes.
12   MS. O'DELL: Object to the form.
13 BY MR. ZELLERS:
14   Q. What was your methodology for discounting the
15 effect of recall bias in the population-based
16 case-control studies?
17   A. My methodology was to rely on a skilled
18 epidemiologist like Dr. Schildkraut to work her way
19 through all of the data and come up to her
20 conclusions.
21   Q. Is there a rate of error in such a
22 methodology?
23   MS. O'DELL: Object to the form.
24   THE WITNESS: I'm not sure I know what
25 you mean by "rate of error."

Page 165

1 BY MR. ZELLERS:
2   Q. Didn't the cohort studies involve a much
3 greater number of women than the case-control studies?
4   A. More women altogether, but less cancer cases.
5   Q. What was your methodology for weighing the
6 power of the cohort of studies versus the case-control
7 studies?
8   A. My methodology was to look at the issues
9 regarding cohort studies that are at fault, that are
10 defective in their trial design and the reporting of
11 their data.
12   Q. You're speaking about cohort studies in
13 general; is that right?
14   A. Well, three cohort studies.
15   Q. Is that right? But you're talking about the
16 studies in general as opposed to specific aspects of
17 the individual cohort studies?
18   A. We can go through the specifics of these
19 three studies.
20   Q. Well, Gates 2010, the Nurses' Health Study,
21 did you review that?
22   A. Yes.
23   Q. It was a follow-up to the cohort study Gertig
24 2000; is that right?
25   A. Yes.

Daniel L. Clarke-Pearson, M.D.

Page 166

1   Q. It's an analysis of data collected in the
2 Nurses' Health Study; correct?
3   A. Yes.
4   Q. The analysis included over 100,000 women; is
5 that right?
6   A. I believe so.
7   Q. The women in the Nurses' Health Study were
8 followed from 1976 to 2006, so for 30 years; is that
9 right?
10   A. The knowledge in this study by the study --
11 the researchers doing the study did not gain any
12 information about exposure until 1982.
13   Q. After following over 100,000 women for three
14 decades, the data did not show a statistically
15 significant relationship between talcum powder use and
16 any type of epithelial ovarian cancer; is that
17 correct?
18   MS. O'DELL: Object to the form.
19   THE WITNESS: That's correct, and
20 there's many defects in the design of this study.
21   For example, the patients were never asked,
22 once again after 1982, whether they used or didn't use
23 talc or how frequently they used talc.
24 BY MR. ZELLERS:
25   Q. Well, let me ask you questions about that.

Page 167

1   The Nurses' Health Study participants were
2 between the ages of 30 to 55 at the start of the study
3 in 1976; is that right?
4   A. I believe so.
5   MS. O'DELL: If you need to see it --
6   THE WITNESS: I don't have -- well,
7 maybe I do have it here.
8 BY MR. ZELLERS:
9   Q. If you need to take a look at it -- do you
10 have it in front of you? I can give it to you if you
11 need it.
12   A. Okay.
13   Q. So my question is the Nurses' Health Study
14 participants were between the ages of 30 to 55 at the
15 start of the study in 1976; is that right?
16   A. Yes.
17   Q. They were asked about their talcum powder use
18 in 1982; is that right?
19   A. That's my understanding, yes.
20   Q. So they would have been between the ages of
21 36 and 61 when they were asked about their talcum
22 powder use; is that right?
23   A. Yes.
24   Q. Most women, as we have discussed, who used
25 talc in their perineal region start that use before

Page 168

1 age 30; right?
2   A. That's what we've seen in other studies.
3   Q. So if a study asks women ages 36 to 61 if
4 they use talcum powder, it would capture the majority
5 of women who use genital powder during the follow-up
6 period; correct?
7   MS. O'DELL: Objection to form.
8   THE WITNESS: During the follow-up
9 period?
10 BY MR. ZELLERS:
11   Q. Yes.
12   A. No. It's a point in time. The question was
13 ever used up to 1982.
14   Q. It would capture the majority of women who
15 use, genital powder use; is that right? In this
16 study.
17   MS. O'DELL: Object to the form.
18   THE WITNESS: Up till 1982.
19 BY MR. ZELLERS:
20   Q. Houghton, 2014, the Women's Health Initiative
21 Study, did you review that study?
22   A. I did.
23   Q. That study involves over 61,000 women; is
24 that right?
25   A. And only 429 cases of ovarian cancer.

Page 169

1   Q. Houghton 2014 did not find a statistically
2 significant relationship between perineal talc use and
3 ovarian cancer among women who had ever used talc; is
4 that right?
5   A. Yes. And this study was not powered to
6 identify --
7   MS. O'DELL: If you need it.
8   THE WITNESS: -- the relative risk that
9 we're talking about in the cohort studies -- I mean
10 the case-control studies. Excuse me.
11 BY MR. ZELLERS:
12   Q. Or among women who had fewer than nine years
13 of perineal talc use; right?
14   A. That's what I believe.
15   Q. I'm looking at page 4, Houghton 2014,
16 Table 2.
17   A. Okay. The question again? Table 2?
18   Q. Yeah. The question is Houghton did not find
19 a statistically significant relationship between
20 perineal talc use and ovarian cancer among women who
21 had fewer than nine years of perineal talc use; right?
22   A. Yes. That sort of exposure is minimal.
23   Q. Or among women who had more than ten years of
24 perineal talc use; is that right?
25   A. Yes.

Daniel L. Clarke-Pearson, M.D.

Page 170

1   Q. And the same results for talcum powder on a
2 sanitary napkins or diaphragms; is that right?
3   A. Yes.
4   Q. Isn't it true that, when combined in a
5 meta-analysis, these cohort studies, the three that
6 we're talking about, have sufficient power to detect a
7 relative risk of 1.25?
8   A. I'm not aware that that -- how that
9 calculation was made.
10   Q. Did you consider the published power
11 calculation by Berge?
12   And so if you look at the Berge 2017 paper,
13 page 6, second column, first paragraph, Berge and his
14 coauthor states (as read):
15   "The statistical power of the
16   meta-analysis of these cohort
17   studies" --
18   MS. O'DELL: I'm sorry, Mike. Where
19 are you reading? Page 6?
20   MR. ZELLERS: Page 6, second column,
21 first paragraph.
22   MS. O'DELL: Thank you.
23   MR. ZELLERS: Sure.
24   THE WITNESS: Second column. That's
25 what this looks like to me (indicating).

Page 171

1 BY MR. ZELLERS:
2   Q. Looking at Exhibit 22.
3   A. I've got 23, which is the more recent paper.
4   Q. Well, take a look at 22, which is the year
5 before, 2017. And I'm looking at page 6. And I'm
6 looking at the last part of the first full paragraph
7 in the right-hand column.
8   Are you with me?
9   A. "The important feature of the present
10 meta-analysis"?
11   Q. Yes.
12   A. Okay.
13   Q. And so if we go down about two-thirds of the
14 way, Berge and the authors conclude (as read):
15   "The statistical power of the
16   meta-analysis of these cohort
17   studies to detect a relative risk
18   of 1.25, similar to the result of
19   the meta-analysis of case-control
20   studies, was 0.99. Thus low power
21   of cohort studies cannot be
22   invoked as an explanation of the
23   heterogeneity of results."
24   Do you see that?
25   A. Let me read it one more time, please.

Page 172

1   Q. Sure.
2   A. So he is saying that the cohort studies are
3 not powered to detect 1.25.
4   Q. What he is saying, I believe, is that the
5 cohort studies are powered to detect a relative risk
6 of 1.25, which was the basis for his conclusion in the
7 last sentence (as read):
8   "Thus low power of cohort studies
9   cannot be invoked as explanation
10   of the heterogeneity of results."
11   MS. O'DELL: Object to the form.
12   THE WITNESS: I read that with a
13 different understanding.
14   What he's saying is that the ability of the
15 cohort study is to detect a relative risk of 1.25 that
16 is similar to the results of the meta-analyses
17 case-control studies was only .99.
18   So those cohort studies aren't powered to
19 detect 1.25.
20 BY MR. ZELLERS:
21   Q. Does Berge conclude "Thus low power of cohort
22 studies cannot be invoked as explanation of the
23 heterogeneity of results"?
24   A. And I'm not sure what I mean -- what you mean
25 by -- what he means by "heterogeneity of results."

Page 173

1   Q. Did I read it correctly?
2   A. Yes, you read it correctly.
3   Q. All right.
4   You're familiar with the hospital-based
5 case-control studies; is that right?
6   A. They are part of the case-control studies,
7 yes.
8   Q. You agree with me that none of the
9 hospital-based case-control studies show a
10 statistically significant association between talc use
11 and ovarian cancer; is that right?
12   MS. O'DELL: Object to the form.
13   THE WITNESS: I would have to go back
14 to each one of those studies, sir.
15 BY MR. ZELLERS:
16   Q. Well, let's -- do you have Langseth there?
17 That might be an easy way to --
18   A. I do.
19   Q. -- take a look at this.
20   We looked at the Langseth as Deposition
21 Exhibit 21.
22   A. I have it.
23   Q. And if we look at his table on page 359, he
24 lists out each of the hospital-based case-control
25 studies.

Daniel L. Clarke-Pearson, M.D.

Page 174

1   Do you see that?
2   A. Right. Those are in the forest plot, yes.
3   Q. None of the hospital-based case-control
4 studies show a statistically significant association
5 between talc use and ovarian cancer; correct?
6   A. Yes.
7   Q. The results of the hospital-based
8 case-control studies are not consistent with the
9 results of the population-based case-control studies;
10 correct?
11   A. That's right. That's why they're combined.
12   Q. What methodology did you use to account for
13 this lack of consistency between the population-based
14 case-control studies and the hospital-based
15 case-control studies?
16   A. This is what the beauty of a meta-analysis
17 is, where it brings together all the studies and comes
18 to a conclusion. And the conclusion here is that
19 there's a 1.35 risk of developing ovarian cancer in
20 women who receive perineal talc.
21   Q. Which Langseth and the other authors
22 concluded was "insufficient to establish a causal
23 association between perineal use of talc and ovarian
24 cancer risk"; correct?
25   MS. O'DELL: Object to the form.

Page 175

1   THE WITNESS: It's statistically
2 significant, which to a clinician means that we could
3 reduce the risk of ovarian cancer if we eliminated
4 talcum powder from the patients that are being exposed
5 to it.
6   MS. BOCKUS: Object. Nonresponsive.
7   MR. ZELLERS: Joined.
8 BY MR. ZELLERS:
9   Q. Are you familiar with the term "selection
10 bias"?
11   A. Yes.
12   Q. What does "selection bias" mean?
13   A. Means that the selection of the patients in a
14 particular study may be inappropriate, that they may
15 not be the proper controls or the proper candidates to
16 be included in the study.
17   Q. You agree that hospital-based case-control
18 studies may be less susceptible to selection bias than
19 population-based case-control studies; correct?
20   MS. O'DELL: Object to the form.
21   THE WITNESS: I'm not sure I believe
22 that.
23 BY MR. ZELLERS:
24   Q. Well, hospital-based case-control studies,
25 you're more likely to be comparing hospitalized

Page 176

1 patients to hospitalized patients; is that right?
2   A. Yes.
3   Q. Whereas in a population-based study, you're
4 more likely to be comparing ill people to healthy
5 people; is that right?
6   MS. O'DELL: Object to the form.
7   THE WITNESS: In a hospital-based
8 study, the people are ill. That's why they're in the
9 hospital.
10 BY MR. ZELLERS:
11   Q. And they're compared to other ill people,
12 other hospitalized patients; is that right?
13   A. Yes.
14   Q. There's a difference in the populations that
15 are being studied between a hospital-based
16 case-control study and a population-based case-control
17 study; correct?
18   A. Yes.
19   Q. How did you account for selection bias in
20 population case-control studies?
21   A. I think if there was selection bias -- and
22 I didn't control for selection bias, but if there was
23 selection bias, first of all, it would be usually
24 negated by the large number of patients in that study.
25   Q. Even among the population-based case

Page 177

1 controls, some studies have shown statistically
2 significant findings and some have not; is that right?
3   A. Yes.
4   Q. What is your methodology for weighing the
5 lack of consistency in statistical significance across
6 case-control studies?
7   MS. O'DELL: Objection to form.
8   THE WITNESS: That's where a
9 meta-analysis becomes a very valuable tool.
10 BY MR. ZELLERS:
11   Q. You agree that, if a study does not show a
12 statistically significant association, it could mean
13 that no risk exists; is that right?
14   A. It's a possibility, yes.
15   MS. O'DELL: Excuse me, Mike. When you
16 get to a -- we've been going an hour and 45 minutes or
17 so.
18   MR. ZELLERS: Let's take a break.
19   THE VIDEOGRAPHER: Going off the record
20 at 12:46 p.m.
21   (Recess taken from 12:46 p m. to 1:45 p.m.)
22   THE VIDEOGRAPHER: Back on record at
23 1:45 p.m.
24 BY MR. ZELLERS:
25   Q. Dr. Clarke-Pearson, in your report, page 7,

Daniel L. Clarke-Pearson, M.D.

Page 178

1 you have a table where you state that you reviewed six
2 meta-analyses reported between 1995 and 2018; is that
3 right?
4   A. Yes. I overlooked adding Berge to this list.
5   Q. What other studies did you overlook adding to
6 this list?
7   A. Subsequent to my report, there's also a
8 meta-analysis by Taher.
9   Q. Any other studies that you omitted from your
10 report and specifically the table on page 7?
11   MS. O'DELL: Object to the form.
12   THE WITNESS: No, not that I'm aware
13 of.
14 BY MR. ZELLERS:
15   Q. What's the difference -- well, strike that.
16   In your report, page 7, you list out five
17 meta-analyses and a pooled analysis; is that right?
18   A. Yes.
19   Q. What is the difference between a pooled
20 analysis and a meta-analysis?
21   A. You know, I really can't give you a good
22 definition of that.
23   Q. How did you select these five studies to set
24 forth in your report?
25   A. I think these were all of the meta-analyses

Page 179

1 that I was aware of.
2   Q. Did you only review the studies that showed a
3 statistically significant relationship between
4 perineal talc use and ovarian cancer?
5   A. I believe I included all the meta-analyses
6 that I could identify.
7   Q. Meta-analyses and pooled analyses combine the
8 work of other published studies into one study; is
9 that right?
10   A. Yes.
11   Q. If there are biases and confounding in the
12 underlying studies, the meta-analysis or pooled
13 analysis will reflect the biases and confounding;
14 correct?
15   MS. O'DELL: Object to the form.
16   THE WITNESS: It obviously varies from
17 one study to another. I would be very surprised if
18 all studies included in the meta-analysis had the same
19 errors, if you will.
20 BY MR. ZELLERS:
21   Q. Well, can you answer that question?
22   If there are biases and confounding in the
23 underlying studies, the meta-analysis or pooled
24 analysis will reflect the biases and confounding;
25 correct?

Page 180

1   MS. O'DELL: Object to the form.
2   THE WITNESS: To some degree.
3 BY MR. ZELLERS:
4   Q. A proper meta-analysis or pooled analysis
5 must analyze the sources of heterogeneity across the
6 studies; right?
7   A. Yes.
8   Q. And a proper meta-analysis or pooled analysis
9 must examine the methodology that lead to the
10 underlying studies; right?
11   A. Yes. I think that's where the weighting done
12 in the meta-analysis helps.
13   Q. Did you examine the methodology in the
14 studies underlying these meta-analyses and pooled
15 analyses?
16   A. Not in detail.
17   Q. Do you agree that consistency exists when
18 different studies look at different populations --
19 strike that. Let me ask that question again.
20   Do you agree that consistency exists when
21 different studies looking at different populations
22 reach consistent results?
23   MS. O'DELL: Object to the form.
24   THE WITNESS: Yes. It seems to be what
25 I would consider consistency.

Page 181

1 BY MR. ZELLERS:
2   Q. A meta-analysis does not demonstrate whether
3 similar results were replicated across different
4 populations; correct?
5   A. Yes. It combines all the papers that were
6 considered in the meta-analysis.
7   Q. It combines study results into one risk
8 calculation; is that right?
9   A. After weighting the different studies in
10 terms of the number of patients and the statistics.
11   Q. Therefore, meta-analyses themselves cannot
12 demonstrate consistency of results across different
13 populations; correct?
14   MS. O'DELL: Object to the form.
15   THE WITNESS: They could demonstrate
16 consistency.
17 BY MR. ZELLERS:
18   Q. How could they demonstrate consistency of
19 results across different populations if what they're
20 doing is combining the study results into one risk
21 calculation?
22   MS. O'DELL: Object to the form.
23   THE WITNESS: I don't understand what
24 you mean by them not being able to demonstrate
25 consistency across different populations.

Daniel L. Clarke-Pearson, M.D.

Page 182

BY MR. ZELLERS:

Q. In your report, you claim that Penninkilampi and every meta-analysis before 2018 report a similar increase in the risk of epithelial ovarian cancer with the use of talcum powder; is that right?

A. Yes.

Q. But each of these meta-analyses that you set forth on page 7 of your report use many of the same studies as the other meta-analyses; is that right?

A. Yes. Over time, new case-control studies were added to the meta-analyses.

Q. Well, for instance, Langseth 2008 and Graham 1999 each include all nine of the studies that were included in Gross and Berg 1995; is that right?

MS. O'DELL: Object to the form.

THE WITNESS: I believe --

MS. O'DELL: Did you say Graham '99?

MR. ZELLERS: No, I said Cramer '99.

MS. O'DELL: Okay. I thought you said Graham.

THE WITNESS: It says Graham on the transcription.

MS. O'DELL: So Cramer is what you're referring to, '99?

MR. ZELLERS: Yes. I'll ask that

Page 183

question again if it was unclear.

BY MR. ZELLERS:

Q. For instance, Langseth 2008 and Cramer 1999 each included all nine of the studies that were included in Gross and Berg 1995; correct?

A. I believe so.

Q. Langseth 2008 included all but one of the 14 studies that were included in Cramer 1999; correct?

MS. O'DELL: And if you need to compare --

THE WITNESS: I need to see the paper. I have Langseth; if I can see Cramer's.

BY MR. ZELLERS:

Q. Well, did you consider this in terms of analyzing the information and data?

A. No.

Q. Take a look, then, if you need to, at the Cramer 1999 paper.

MS. O'DELL: Just a moment. I'm sorry.

BY MR. ZELLERS:

Q. We're still just looking at your folders from earlier today that you have in front of you; right, Doctor?

A. Yes.

Q. Let me phrase it a different way, and then we

Page 184

can let the record -- correct this later if need be.

Doctor --

MS. O'DELL: I'll have it in front of you in one moment, Doctor.

BY MR. ZELLERS:

Q. Okay. Dr. Clarke-Pearson, you now have Langseth 2008 and Cramer 1999 in front of you; is that right?

A. Yes.

Q. Langseth 2008 included all but one of the 14 studies that were included in Cramer 1999; is that right?

A. This is the Cramer case-control study.

Q. Let me ask you the question this way, Doctor: Do you have any reason to doubt as you sit here or dispute as you sit here that Langseth 2008 did not include all but one of the 14 studies that were included in Cramer 1999?

A. I would accept that as the truth.

Q. Thank you. As you sit here, do you have any reason to doubt or dispute that Langseth 2008 included all but one of the 15 studies that were included in Huncharek 2003?

I understand you don't have the studies in front of you to be able to make that --

Page 185

MS. O'DELL: Let me just -- I would just object to the line of questions. If you're going to ask the specific studies that are listed in the table and ask him to compare --

MR. ZELLERS: No. What I'm asking him, Counsel --

MS. O'DELL: Let me finish.

It's unfair to ask him to make comparisons regarding the studies included in the meta-analyses without affording him the opportunity to look at the articles themselves.

MR. ZELLERS: And, Counsel, as you know, we've got limited time, and I don't want to sit here --

MS. O'DELL: It's still an unfair question.

MR. ZELLERS: It is not an unfair question to ask this witness if he has any reason as he sits here to dispute or to doubt that Langseth 2008 included all but one of the 15 studies that were included in Huncharek 2003.

MS. O'DELL: Well, that's not a fair question when you're not providing him an opportunity to compare the two.

And so if Dr. Clarke-Pearson wants to see a

Daniel L. Clarke-Pearson, M.D.

Page 186

1 copy of the study, then we'll put it in front of him,
2 because that's not a fair analysis, particularly when
3 you're talking about multiple -- more than 10 to 15
4 meta-analyses -- excuse me -- cohorts over time.
5   MR. ZELLERS: Counsel, I've asked you a
6 number of times not to make speaking objections. All
7 that I am doing is asking the doctor questions about
8 the studies included in the six meta-analyses and
9 pooled analysis that he sets out in a chart.
10   If he doesn't have the answer, my question
11 is framed as to whether or not he has any reason to
12 dispute or doubt the overlap of studies.
13   MS. O'DELL: Well, I would just say,
14 Dr. Clarke-Pearson, to the degree you remember, you
15 can answer his questions. But, to the degree he asks
16 you to assume something, don't assume that what
17 counsel is stating is correct because it may or may
18 not be true.
19   MR. ZELLERS: And I'm not asking the
20 doctor to assume.
21   MS. O'DELL: Yes, you did.
22   MR. ZELLERS: I did not ask him to
23 assume, Counsel. You can go back and read the
24 question, but it did not ask him to assume that. It
25 asked him if he was aware of there being any

Page 187

1 difference in terms of Langseth including all but one
2 of the 15 studies that were included in Huncharek
3 2003.
4   MS. O'DELL: I stand corrected. You
5 said "Do you have any reason to doubt or dispute,"
6 which I took to be --
7   MR. ZELLERS: "Do you have any reason
8 to" --
9   MS. O'DELL: -- which I took to be
10 assume.
11   And I'm asking you to assume that counsel is
12 not being accurate.
13 BY MR. ZELLERS:
14   Q. Can you answer my question, Doctor?
15   And here's my question: Do you have any
16 reason to believe that Langseth 2008, which you cite,
17 included all but one of the 15 studies that were
18 included in Huncharek 2003, which you cite?
19   A. Without reading and going through the table
20 of the 'teen or so studies, I would have to assume
21 that you're representing properly what --
22   Q. That is not a comparison that you have made
23 personally; correct?
24   A. I have not. And if I did, I can't remember
25 now.

Page 188

1   Q. Okay.
2   A. I mean, if this is a quiz about memorizing
3 details of clinical studies, then...
4   Q. I don't want it to be a quiz. Let me ask you
5 a new question.
6   If the meta-analyses are all combining the
7 same set of studies, you would expect them to yield
8 similar results; correct?
9   A. If they only contain the same set of studies
10 but each one had slightly different, and the more
11 recent ones added studies to them.
12   Q. Have you attempted to quantify how much
13 talcum powder reaches a woman's ovaries when they use
14 a talcum powder product?
15   A. Have I done some experiment?
16   Q. Yes.
17   A. I know that talcum powder gets there; I have
18 not done any experimentation to that question.
19   Q. Do you have any -- were you finished?
20   A. Yes.
21   MS. BOCKUS: Object as nonresponsive.
22 BY MR. ZELLERS:
23   Q. Do you have any idea how much talcum powder
24 reaches a woman's ovaries each time she uses it?
25   A. I'm sure it varies depending upon the

Page 189

1 menstrual cycle, the age of the patient, the patient's
2 anatomy.
3   Q. It's fair to say you don't know and have not
4 done any type of calculation or experiment to
5 determine the answer to that question; correct?
6   MS. O'DELL: Object to the form.
7   THE WITNESS: That's correct.
8 BY MR. ZELLERS:
9   Q. Isn't the biological mechanism dependent on
10 how much talc a woman's ovaries are exposed to?
11   A. Which biological mechanism are you talking
12 about?
13   Q. Dose response.
14   MS. O'DELL: Object to the form.
15   THE WITNESS: So, then, rephrasing your
16 question, isn't the dose response dependent upon how
17 much talc a woman's ovaries are exposed to?
18 BY MR. ZELLERS:
19   Q. I'll accept that.
20   A. That sounds like the answer -- you answered
21 your own question.
22   Q. Well, I need you to answer the question. The
23 answer is a yes to that question; correct?
24   A. The dose is dependent upon how much talc gets
25 to the ovaries, yes.

Daniel L. Clarke-Pearson, M.D.

Page 190

1   Q. And you've not done a calculation or
2 experiment to determine what that amount is; correct?
3   A. That's correct.
4   Q. All right.
5   Let me mark Cramer 2016. We discussed it
6 earlier, but we'll mark it for the record. This is a
7 study that you cite in your materials. We'll mark it
8 as Exhibit 26.
9   (Exhibit No. 26 was marked for identification.)
10 BY MR. ZELLERS:
11   Q. You recognize this paper; correct?
12   A. I've reviewed it.
13   Q. This is a retrospective case-control study
14 published in 2016; correct?
15   A. Yes.
16   Q. You discuss this study in your report on
17 page 9; is that right?
18   A. Let me turn to page 9.
19   Q. Sure. I'm looking under "Biologic
20 Gradient/Dose-response" right in the middle.
21   You claim that (as read):
22   "A number of studies have
23   demonstrated an association
24   between 'dose' and the occurrence
25   of EOC [or epithelial ovarian

Page 191

1   cancer] (response)."
2   Is that right?
3   A. That's correct.
4   Q. Let's look at what the Cramer study shows.
5   Turn to page 337 of the Cramer paper, if you
6 will, Exhibit 26 to the deposition.
7   Do you see Table 1?
8   A. Yes, sir.
9   Q. Table 1 shows the risk of ovarian cancer for
10 women who use talc daily for different periods of
11 time -- 1 year, 1 to 5 years, 5 to 20 years, and more
12 than 20 years. Is that right?
13   A. Yes.
14   Q. There was only statistical significance for
15 one to five years of use and for more than 20 years of
16 use; is that right?
17   A. According to the odds ratio and the
18 confidence intervals, yes.
19   Q. If there is a dose response, shouldn't there
20 continue to be statistical significance with increased
21 exposure?
22   A. In general, you would think that. But, on
23 the other hand, maybe we don't have to have a dose
24 response to cause cancer.
25   Q. Well, certainly you've opined in your report

Page 192

1 that there is a dose response; is that right?
2   A. Yes.
3   Q. And, in fact, at least looking at Table 1 of
4 the Cramer study, this does not show a dose response;
5 correct?
6   MS. O'DELL: Object to the form.
7   THE WITNESS: So, going down that
8 table, there is more of a dose response as we get
9 under the second half of that table, toward "general
10 talc applications."
11 BY MR. ZELLERS:
12   Q. There is not a consistent dose response;
13 correct?
14   A. Not a consistent.
15   Q. Yes. I mean, you get a statistically
16 significant finding and then a period of time where
17 there's not a statistically significant finding and
18 then another period of time where there is a
19 statistically significant finding; is that right?
20   MS. O'DELL: Object to the form.
21   THE WITNESS: As I read through the
22 second half of this table, there's a consistent
23 statistically significant finding beginning after less
24 than 360 applications, equivalent to one year of daily
25 use.

Page 193

1 BY MR. ZELLERS:
2   Q. Well, when you review, you consider all of
3 the data; correct?
4   A. Yes.
5   Q. The top of the Table 1 is not consistent with
6 the bottom of Table 1, at least in terms of
7 statistically significant findings; is that right?
8   A. The two -- the two vary, depending upon how
9 you quantitate dose.
10   Q. Another criteria or factor for Bradford Hill
11 is biological plausibility; is that right?
12   A. Yes.
13   Q. The biological mechanisms of cancer are not
14 your area of expertise; is that correct?
15   MS. O'DELL: Object to the form.
16   THE WITNESS: I think, as a gynecologic
17 oncologist, I have a good understanding of the
18 biological mechanisms of cancer. For example, human
19 papillomavirus causes cervical cancer, vaginal cancer,
20 vulvar cancer, anal cancer, oropharyngeal cancer.
21 BY MR. ZELLERS:
22   Q. Do you defer to other experts on the topic of
23 biologic plausibility?
24   A. I think there are some that know more than
25 I know about it. But I know that, for example, in

Daniel L. Clarke-Pearson, M.D.

Page 194

1 this disease of ovarian cancer caused by talcum
2 powder, inflammation is the most likely cause.
3   Q. And do you consider yourself to be an expert
4 on the topic of biologic plausibility as it relates to
5 talcum powder and ovarian cancer?
6   MS. O'DELL: Objection to form. Asked
7 and answered.
8   THE WITNESS: I think I have a very
9 good understanding of that, and I'm not sure how you
10 define an expert.
11 BY MR. ZELLERS:
12   Q. Is all epithelial ovarian cancer caused by
13 the same mechanism?
14   A. I don't think so.
15   Q. You stated before that there are different
16 mechanisms; is that right?
17   A. I said -- yes.
18   Q. What is the biologic mechanism for serous
19 ovarian cancer?
20   A. There could be several biological mechanisms
21 for any of the ovarian cancers.
22   Q. Well, what biologic mechanisms are there,
23 based upon your experience, for serous cancer --
24 ovarian cancer?
25   A. One of the biologic mechanisms are BRCA1 to 2

Page 195

1 mutations. And, as I discussed previously, all
2 cancers are caused by mutations of genes that regulate
3 cell growth and result in invasion and metastases.
4   Q. Any others?
5   A. Anything else beside gene mutations?
6   Q. Gene mutations, yes, for serous ovarian
7 cancer.
8   A. There are always gene mutations causing the
9 cancer. And, therefore, if you're just specifically
10 talking about serous cancers, then gene mutations for
11 all serous cancers occur. They are not normal cells.
12   Q. Does talcum powder increase all subtypes of
13 ovarian cancer?
14   MS. O'DELL: Objection. Asked and
15 answered.
16   THE WITNESS: I think the epidemiologic
17 data would suggest that serous cancers are the most
18 common but endometrioid are there.
19   And the other study -- other types of
20 epithelial ovarian cancers -- clear cell and
21 mucinous -- are so infrequent -- they're rare cancers.
22 And, therefore, we don't have statistical power to
23 decide whether they're caused by talc or not.
24 BY MR. ZELLERS:
25   Q. Different subtypes of epithelial ovarian

Page 196

1 cancer have different biological mechanisms; correct?
2   A. Again, I'm not sure what you mean by
3 "biological mechanism."
4   Q. You're not familiar with biological
5 mechanisms that cause ovarian cancer?
6   A. The biological mechanism that I've been
7 trying to explain to you is gene mutation.
8   Q. That's the only biological mechanism that
9 causes ovarian cancer, in your experience; is that
10 right?
11   A. You're talking about what causes ovarian
12 cancer, not the mechanism that becomes ovarian cancer
13 or what ovarian cancer represents.
14   Q. I'm asking you the mechanism that causes
15 ovarian cancer. And you have told me that, with
16 talcum powder, it is gene mutation; is that right?
17   MS. O'DELL: Object to the form.
18   THE WITNESS: As it is for all cancers.
19 As it is for all ovarian cancers.
20 BY MR. ZELLERS:
21   Q. If talc is associated with all subtypes of
22 epithelial ovarian cancer or with different subtypes
23 in different studies, doesn't that suggest that the
24 association is by chance?
25   MS. O'DELL: Object to the form.

Page 197

1   THE WITNESS: So no carcinogen is going
2 to cause cancer in every circumstance in every
3 patient. Some patients may be more susceptible to a
4 carcinogen; others may be more resistant.
5   Women with BRCA1 mutations don't always
6 develop ovarian cancer, but they are at much higher
7 risk. It usually causes -- it requires a number of
8 mutations before a malignancy occurs, not just one.
9 BY MR. ZELLERS:
10   Q. You would agree that different studies have
11 found different associations between talcum powder use
12 and different types of epithelial ovarian cancer; is
13 that right?
14   A. The -- yes, and because possibly many of
15 those rare cancers, like mucinous cancers and clear
16 cell cancers, are not -- the studies aren't powered to
17 identify those. So we don't know, I guess would be my
18 answer.
19   Q. Putting aside inhalation for the moment, your
20 opinion is that talcum powder travels from the
21 perineal region to the ovaries through the woman's
22 reproductive tract; is that right?
23   A. Yes, sir.
24   Q. So the talcum powder must travel across the
25 vulva, through the labia majora, through the labia

Daniel L. Clarke-Pearson, M.D.

Page 198

1 minora, across the -- and clitoris, across the
2 perineal body, up into the vagina, into the cervical
3 canal, through the cervix and cervical mucosa, or
4 mucus, into the endometrial cavity, through the
5 uterus, into the fallopian tube opening, across the
6 entire length of the fallopian tube to the fimbria,
7 and then into the ovary; is that right?
8   A. Yes, sir.
9   Q. If talcum powder can make this migration, can
10 other substances also make the same migration?
11   A. I presume so.
12   Q. Sand from the beach?
13   A. I think the particle size may have some
14 bearing on how far it can get up the reproductive
15 tract.
16   Q. Toilet paper particles?
17   MS. O'DELL: Object to the form.
18   THE WITNESS: Again, depends upon the
19 particle size.
20 BY MR. ZELLERS:
21   Q. There is no human study that demonstrates the
22 migration of any particulate matter from the perineum
23 to the ovaries; correct?
24   MS. O'DELL: Object to the form.
25   THE WITNESS: Number of studies that

Page 199

1 show that once it's in the vagina, it can migrate --
2 BY MR. ZELLERS:
3   Q. There is --
4   A. -- to the ovary.
5   Q. But the answer to my question is correct.
6 There are no human studies that demonstrate the
7 migration of any particulate matter from the perineum
8 to the ovaries; correct?
9   MS. O'DELL: Object to the form.
10   THE WITNESS: Nobody has studied it
11 that I'm aware of.
12 BY MR. ZELLERS:
13   Q. None of the articles you cite in your report
14 actually looked at whether talc can migrate from
15 perineal application through the fallopian tubes to
16 the ovaries; correct?
17   MS. O'DELL: Object to the form.
18   THE WITNESS: Well, if you go to
19 Langseth, for example, on the second page underneath
20 the forest plot at the end of the second full
21 paragraph -- I'm sorry. I've got your exhibit.
22 BY MR. ZELLERS:
23   Q. Well, you have the exhibit. I should have a
24 copy.
25   A. Okay.

Page 200

1   Q. And my question to you is --
2   MS. O'DELL: I think he was finished --
3 he wasn't finished.
4   THE WITNESS: I was going to read this
5 to you from Langseth. And the sentence says
6 (as read):
7   "The evidence of talc migrating to
8   the ovaries lends credibility to
9   such a possible association."
10 BY MR. ZELLERS:
11   Q. Can you answer my question?
12   A. I was reporting to you a study.
13   Q. I need you to answer my question if you can.
14   A. Okay.
15   Q. I'll ask it again.
16   Is there any human study that demonstrates
17 the migration of any particulate -- and let me
18 withdraw that, because I think I moved on to the next
19 question.
20   None of the articles that you cite actually
21 looked at whether talc can migrate from the perineal
22 application through the fallopian tubes to the
23 ovaries; correct?
24   MS. O'DELL: Object to the form.
25   THE WITNESS: That's correct.

Page 201

1 BY MR. ZELLERS:
2   Q. All right. You also cannot cite any article
3 that shows granulomas, fibrosis, or adhesions anywhere
4 up the reproductive tract of a woman as a result of
5 her external genital talc application, can you?
6   MS. O'DELL: Object to the form.
7   THE WITNESS: No.
8 BY MR. ZELLERS:
9   Q. Let's talk about the studies that you cite in
10 your report in support of your theory of migration.
11   MS. O'DELL: Object to -- excuse me.
12 Sorry.
13   MR. ZELLERS: It's okay.
14   MS. O'DELL: I apologize.
15 BY MR. ZELLERS:
16   Q. In support of your theory of migration, you
17 discuss sperm. I'm looking at page 7, last paragraph
18 that carries over onto page 8. Is that right?
19   A. I have it.
20   MS. O'DELL: Object to form.
21 BY MR. ZELLERS:
22   Q. Sperm have tails and motility; is that right?
23   A. Yes, and that's acknowledged in my report.
24   Q. Sperm affirmatively move themselves up the
25 reproductive tract; is that right?

Daniel L. Clarke-Pearson, M.D.

Page 202

1   A. They can.
2   Q. You cite Egli, 1961, the carbon particle
3 study. Are you familiar with that, or do you need me
4 to hand you another copy?
5   A. I've reviewed it before. It's been a little
6 while.
7   Q. Well, let me ask you a couple of questions.
8   A. Sure.
9   Q. And if you need the study, then I'll be happy
10 to have you take a look at it.
11   Egli did not involve talcum powder; correct?
12   A. No. These are carbon particles.
13   Q. Egli used carbon particles that were
14 suspended in a solution that had the consistency of
15 seminal fluid; is that right?
16   MS. O'DELL: If you need to take a
17 moment to review, Doctor, feel free to do that.
18   THE WITNESS: They were suspended in
19 dextran suspension.
20 BY MR. ZELLERS:
21   Q. Is that seminal fluid, fluid that sperm are
22 suspended in?
23   A. No.
24   Q. What solution were they suspended in?
25   A. Dextran.

Page 203

1   Q. What support do you have for the proposition
2 that talcum powder behaves similarly to carbon
3 particles suspended in a dextran fluid-like substance?
4   A. I think it's very similar to talcum powder
5 particles progressing up. Dextran is a thick,
6 glucose-rich medium that is much like vaginal fluid,
7 if you will.
8   Q. It's a fluid; right?
9   A. Yes.
10   Q. Talcum powder is a particle; correct?
11   A. Once talcum powder gets into the vagina, it
12 becomes part of the vaginal fluid.
13   Q. The Egli study involved three women; is that
14 right?
15   A. Yes.
16   Q. Tiny sample size; correct?
17   A. Yes.
18   Q. They used intramuscular oxytocin to aid the
19 transport of the particles; is that right?
20   A. Yes. It stimulated the uterus to contract.
21   Q. And for the administration of the carbon
22 particles, the women were laying on their backs with
23 their heads tilted at a downward angle; is that right?
24   A. That's what it says.
25   Q. Do you agree that laying down with their

Page 204

1 heads tilted downward is a very -- is very different
2 from the way in which women generally apply talcum
3 powder to their perineal region?
4   A. Honestly, I don't know how they apply talcum
5 powder to their perineal region. I would imagine
6 they're not with their head down, but they may be
7 sitting, they may be standing, they may be lying.
8   Q. Based upon your experience, it's different;
9 correct?
10   A. I don't have any experience with talcum
11 powder application.
12   Q. Right. So you don't know whether or not most
13 women apply talcum powder to their perineal region
14 with their head toward the ground and their legs up in
15 the air?
16   MS. O'DELL: Object to the form.
17   THE WITNESS: I think it's unlikely
18 that they have their heads to the ground and legs in
19 the air, but they have probably multiple positions
20 they could apply it in.
21 BY MR. ZELLERS:
22   Q. Even with these artificial conditions, the
23 researchers only found carbon particles in the
24 fallopian tubes of two of the three women; is that
25 right?

Page 205

1   A. I think that's what the results said.
2   Q. Are you familiar with the Venter 1979 study
3 that you cite?
4   A. I'll have to pull it back out to refresh my
5 memory. It's been a few months since I looked at
6 that.
7   Q. Well, can I ask you a few questions about it?
8   A. If I can answer them, I will. Sure.
9   Q. Is this the radioactive marker study?
10   A. Yes.
11   Q. That study did not involve talcum powder; it
12 involved a particle with a radioactive tracer. Is
13 that right?
14   A. Yes. Technetium albumin in microspheres.
15   Q. What support do you have for the proposition
16 that talcum powder behaves similarly to this kind of
17 particle?
18   A. I think that talcum powder is similar to
19 these particles. It's small and can migrate.
20   Q. In the study it involved a small sample size;
21 right? Only 24 women?
22   MS. O'DELL: Object to the form.
23   THE WITNESS: Yes.
24 BY MR. ZELLERS:
25   Q. The women laid on their backs with their

Daniel L. Clarke-Pearson, M.D.

Page 206

1 buttocks elevated; is that right?

2   A. When it was applied, and then the patients

3 didn't undergo surgery until the next day. So the

4 patients, after being in the position where the

5 talc -- where the radioactive tracer was applied, were

6 then up and about until they came in for surgery the

7 next day. So they were in different positions.

8   Q. Is that really what you think, based upon

9 your review of the study?

10   A. You don't think that the patient was laying

11 in bed for 24 hours until she had surgery?

12   Q. Doctor, your recollection of this study is

13 that the radioactive tracer marker was used and then

14 the women were up and around?

15   MS. O'DELL: Object to the form.

16 BY MR. ZELLERS:

17   Q. In fact, after the radioactive marker was

18 administered, the women remained laying in the

19 position with their -- on their backs with their

20 buttocks elevated for two hours, with their legs

21 pressed together; is that right?

22   A. I would have to find it to refresh my memory.

23   Q. If that's true, that would be different than

24 your understanding of how women use talcum powder in

25 the genital area; correct?

Page 207

1   MS. O'DELL: Objection. Misstates the

2 doctor's testimony.

3   If you need to review --

4   THE WITNESS: Again, I don't think that

5 we know -- I know how women apply talcum powder. But

6 these women didn't lay supine for 24 hours until they

7 had their surgery, when they found the radioactive

8 microspheres in the ovary.

9 BY MR. ZELLERS:

10   Q. Do you know whether or not they laid supine

11 for two hours after the radioactive marker was

12 administered with their legs pressed together?

13   A. Yes.

14   Q. Yes, you agree with that; correct?

15   A. Yes.

16   Q. And even under these artificial conditions,

17 the researchers only found radioactive activity in the

18 fallopian tubes or ovaries of 9 of the 21 women; is

19 that right?

20   MS. O'DELL: Object to the form.

21   THE WITNESS: That's what they reported

22 in 24 hours.

23 BY MR. ZELLERS:

24   Q. You cite Sjosten, 2004, the glove study; is

25 that right?

Page 208

1   A. I did.

2   Q. That study did not involve talcum powder; it

3 involved starch. Is that right?

4   A. Yes.

5   Q. Sjosten involved the researchers examining

6 the women's cervix with their fingers; is that right?

7   Are you able to answer that question?

8   A. I need to read along with you.

9   So they examined -- they did a pelvic exam,

10 a bimanual exam on the patients.

11   Q. Examining the women's cervix with their

12 fingers; is that correct?

13   A. And examining the vagina.

14   Q. What is your basis for saying that pressing

15 gloved fingers against the cervix is comparable to an

16 external dusting of talcum powder?

17   MS. O'DELL: Object to form.

18   THE WITNESS: I think it deposits the

19 substance, the powder, against the cervix.

20 BY MR. ZELLERS:

21   Q. And the study found particles in the

22 reproductive tract of women who were examined with

23 powder-free gloves; is that right?

24   A. I believe so.

25   Q. You cite the Heller study of women's ovaries

Page 209

1 after surgical oophorectomy; is that right?

2   A. Yes.

3   Q. Didn't Heller find talc in tissues of all 24

4 patients, including the 12 who did not use perineal

5 talc?

6   A. Give me a moment.

7   Q. Let me try to ask it this way so that we can

8 move on.

9   Do you have any reason to dispute that

10 Heller found talc in tissues of all 24 patients,

11 including the 12 who did not use perineal talc?

12   MS. O'DELL: Object to the form.

13   THE WITNESS: Yes, as long as there's

14 not an issue with recall bias.

15 BY MR. ZELLERS:

16   Q. If talcum powder migrates from the perineal

17 region to the ovaries, shouldn't exposure to talc be

18 far greater in concentration in the rectal, vulvar,

19 vaginal, cervical, and uterine tissues which are

20 closer to the area of initial exposure?

21   MS. O'DELL: Object to the form.

22   THE WITNESS: I'm not sure what the

23 basis of that observation is. The urethra and anus

24 have sphincters. The urethra and anus also have an

25 exit mechanism by urination or defecation.

Daniel L. Clarke-Pearson, M.D.

Page 210

BY MR. ZELLERS:
 2   Q. So you -- I just want to make sure I'm clear.
 3 You disagree that -- if talcum powder migrates from
 4 the perineal region to the ovaries, you disagree that
 5 exposure to talc would be greater in concentration in
 6 the rectal, vulvar, vaginal, cervical, and uterine
 7 tissues; correct?
 8   MS. O'DELL: Objection. Asked and
 9 answered.
10   THE WITNESS: I'm not understanding
11 your question. Would be greater where?
12 BY MR. ZELLERS:
13   Q. Would be greater in the rectal, vulvar,
14 vaginal, cervical, and uterine tissues than in the
15 ovaries.
16   MS. O'DELL: Objection. Asked and
17 answered.
18   THE WITNESS: I don't have any evidence
19 about the rectum or the urethra. And it would be --
20 yes, more likely than not, there would be more on the
21 vulva than on the ovaries. All of it that goes on the
22 vulva does not land on the ovaries.
23 BY MR. ZELLERS:
24   Q. Talc particles should be causing inflammation
25 in all those organs and areas if your theory is

Page 211

 1 correct; is that right?
 2   A. No.
 3   MS. O'DELL: Object to the form.
 4 BY MR. ZELLERS:
 5   Q. Why would you not expect inflammation in the
 6 rectal, vulvar, vaginal, cervical, and uterine
 7 tissues?
 8   MS. O'DELL: Object to the form.
 9   THE WITNESS: So there's no -- no
10 evidence that this talc gets into the rectum that I'm
11 aware of, unless you have some evidence that I'm not
12 seeing.
13 BY MR. ZELLERS:
14   Q. Why do talc particles not cause inflammation
15 in the other organs and areas?
16   A. I think the other organs -- the vagina,
17 cervix, uterus, and fallopian tubes -- are different
18 tissues; and different tissues have different
19 susceptibility, if you will, to the impact of talcum
20 powder and its contents.
21   Q. What is it about the tissues of the vulvar,
22 vaginal, cervical, and uterine areas that would result
23 in talc not causing inflammation to those tissues but
24 causing, at least under your theory, inflammation to
25 the ovary?

Page 212

 1   MS. O'DELL: Object to the form.
 2   THE WITNESS: Because the ovary has a
 3 different epithelium, a different surface. The
 4 vagina -- I'm sorry -- the vulva, vagina, and
 5 exocervix are all squamous epithelium. They are much
 6 more susceptible to HPV. So I can turn around the
 7 explanation and say HPV doesn't infect the
 8 endometrium -- the uterus, fallopian tubes, or
 9 ovaries. So some tissues are more susceptible to a
10 carcinogen than others.
11 BY MR. ZELLERS:
12   Q. What study are you referring to for that
13 proposition?
14   A. About HPV?
15   Q. No. About the tissue being the same --
16 strike that.
17   Tissue being different and not susceptible
18 to inflammation from talc in the human vulvar,
19 vaginal, cervical, and uterine tissues.
20   MS. O'DELL: Object to the form.
21   THE WITNESS: They are all different
22 tissues, and we have not seen any inflammation or
23 cancer associated with talcum powder in those organs.
24 BY MR. ZELLERS:
25   Q. Is there a study that you're referring to

Page 213

 1 that finds that there is not inflammation from talc to
 2 those tissues?
 3   MS. O'DELL: Object to the form.
 4   THE WITNESS: I don't have a study,
 5 but, obviously, it's not associated with cancers of
 6 those tissues.
 7 BY MR. ZELLERS:
 8   Q. There are no studies that show inflammation
 9 as a result of genital talc use result in cancer in
10 those areas; is that right?
11   MS. O'DELL: Objection to form.
12   THE WITNESS: In what areas now are you
13 talking about?
14 BY MR. ZELLERS:
15   Q. Let me make it even simpler.
16   There's no studies that show inflammation as
17 a result of genital talc use in the vulvar, vaginal,
18 cervical, and uterine areas; is that right?
19   A. That's correct.
20   MS. O'DELL: Object to the form.
21 BY MR. ZELLERS:
22   Q. There are no studies that show a link between
23 external genital talc use and rectal, vulvar, vaginal,
24 cervical, or uterine cancer; is that right?
25   A. That's correct.

Daniel L. Clarke-Pearson, M.D.

Page 214

1   Q. In Exhibit B of your report, you include a
2 study published by Huncharek in 2007. That's page 11.
3   Do you recall that study?
4   A. No, but I'd like to refresh my memory.
5   MS. O'DELL: Which Huncharek?
6   MR. ZELLERS: 2007.
7 BY MR. ZELLERS:
8   Q. Do you have that easily available?
9   This is a study that you cite in your
10 materials reviewed; is that right?
11   A. Yes.
12   Q. It's a meta-analysis of studies and the
13 relationship between ovarian cancer and using
14 diaphragms that are dusted with talcum powder; is that
15 right?
16   A. Yes.
17   Q. A diaphragm is inserted directly onto a
18 woman's cervix; is that right?
19   A. Yes.
20   Q. You did not include Huncharek 2007 in your
21 list of meta-analyses regarding talc and ovarian
22 cancer on page 7 of your report, did you?
23   MS. O'DELL: Object to the form.
24   THE WITNESS: No, because it wasn't
25 dealing with applying talcum powder to the vulva,

Page 215

1 perineum.
2 BY MR. ZELLERS:
3   Q. Well, your theory, putting aside inhalation,
4 is that the talcum powder travels from the perineal
5 region through the vagina through the cervix through
6 the uterus and then into the fallopian tubes; is that
7 right?
8   A. Yes.
9   Q. How, then, do you validate excluding data
10 about the relationship between ovarian cancer and
11 talcum powder that is applied directly to the cervix?
12   MS. O'DELL: Object to the form.
13   THE WITNESS: Because it's not the
14 volume of talcum powder that is used on the vulva.
15 And, over a period of time, application of diaphragms
16 is most likely much less likely than somebody using
17 talcum powder on the vulva on a daily basis.
18 BY MR. ZELLERS:
19   Q. On what study are you relying for that
20 statement?
21   A. My clinical experience of understanding the
22 sexual lives of women. They don't use diaphragms
23 every day, in most cases.
24   Q. Are you aware of any study that talcum powder
25 affects the body differently when it is applied to the

Page 216

1 perineal region and travels to the cervix compared to
2 when it is applied directly to the cervix?
3   MS. O'DELL: Object to the form.
4   THE WITNESS: I'm not aware of any
5 study, no.
6 BY MR. ZELLERS:
7   Q. When applied to the perineal region, the
8 talcum powder would also be in close contact with a
9 woman's urethra; correct?
10   A. Yes.
11   Q. Substances are capable of traveling up the
12 urethra; right?
13   A. Not that I know of, except for bacteria.
14   Q. Women get urinary tract infections when
15 bacteria travels up the urethra; right?
16   A. I recognize that as a modal -- motile, like
17 sperm and bacteria, when I discuss lower genital tract
18 migration from the vagina up into the tubes and
19 ovaries with sperm and sexually transmitted infection.
20 So, yes, women get urinary tract infections.
21   Q. Studies do not show an increase in bladder
22 cancer with talcum powder use; is that right?
23   A. That's right. The bladder is a different
24 epithelium than the ovary.
25   Q. And studies do not show an increase in rectal

Page 217

1 cancer with talcum powder use; is that right?
2   A. That's correct.
3   MS. O'DELL: Objection. Asked and
4 answered.
5 BY MR. ZELLERS:
6   Q. Are you opining on inhalation exposure as a
7 plausible mechanism for talcum powder to reach the
8 ovaries, or do you defer to other experts on that?
9   A. I think there's literature that suggests that
10 it's a lower possibility, but inhalation of asbestos
11 can increase the risk of ovarian cancer.
12   Q. Well, you rely in part on Steiling 2018; is
13 that right? This is at page 8 of your report.
14   A. IARC and the Steiling.
15   Q. Right. Steiling 2018 deals generally with
16 cosmetic powders, not talcum powder; correct?
17   A. I need to look at the paper again.
18   Q. Well, either your counsel can hand it to you
19 or I can hand it to you.
20   MR. ZELLERS: Did you find it, Counsel?
21 BY MR. ZELLERS:
22   Q. Do you have the Steiling paper in front of
23 you?
24   A. Yes --
25   MS. O'DELL: Do you have a copy for me,

Daniel L. Clarke-Pearson, M.D.

Page 218

1 please, if you don't mind. Thank you.
2   Are you going to mark that, Mike, or are
3 you --
4   MR. ZELLERS: If you want me to mark
5 it, I can. I think we all know what it is.
6   MS. O'DELL: I'm just asking.
7   MR. ZELLERS: Would you like it marked?
8   MS. O'DELL: Only if you were going to
9 mark it, I was just going to put a number on it.
10   MR. ZELLERS: Well, I just have a few
11 basic questions.
12 BY MR. ZELLERS:
13   Q. So, Doctor, my first question is the Steiling
14 2018 deals generally with cosmetic powders, not talcum
15 powder specifically; is that right?
16   A. Apparently so, yes.
17   Q. And Steiling 2018 just discusses the fact
18 that particles can be inhaled; is that right?
19   A. Yes.
20   MS. O'DELL: Object to the form.
21 BY MR. ZELLERS:
22   Q. It says nothing about inhaled particles
23 migrating to the ovaries, does it?
24   A. No.
25   Q. In fact, it says nothing about inhaled

Page 219

1 particles migrating anywhere, does it?
2   MS. O'DELL: Objection.
3   THE WITNESS: It doesn't talk about
4 migration. You're right.
5 BY MR. ZELLERS:
6   Q. And it also says nothing about inhaled
7 particles causing ovarian cancer; is that right?
8   A. In this particular study, although we know
9 from asbestos studies that it does.
10   Q. Well, don't studies of talcum powder use fail
11 to show statistically significant association between
12 nongenital use of talcum powder and ovarian cancer?
13   A. I believe so.
14   Q. If inhaled talc could migrate to the ovaries,
15 wouldn't you expect to see increased ovarian cancer
16 risk with nongenital use of talcum powder?
17   MS. O'DELL: Object to the form.
18   THE WITNESS: In other words, inhaled.
19 I think the inhalation is much smaller, but, to date,
20 we haven't seen an increased risk of ovarian cancer.
21 BY MR. ZELLERS:
22   Q. With inhaled talcum powder; correct?
23   A. With inhaled talcum powder.
24   Q. And that was a finding that you read about in
25 Cramer 2016 as well as other places; correct?

Page 220

1   MS. O'DELL: Object to the form.
2 BY MR. ZELLERS:
3   Q. I'll withdraw the question and move on.
4   Do you agree -- well, strike that.
5   You assert that talcum powder, when it
6 reaches the ovaries, it elicits an inflammatory
7 response that is linked to ovarian cancer; is that
8 right?
9   A. Yes. I think that's the mechanism by which
10 gene mutation occurs.
11   Q. Is it your opinion -- strike that.
12   Is your opinion related to all of the
13 different histologic types of epithelial ovarian
14 cancer?
15   MS. O'DELL: Objection. Asked and
16 answered.
17   THE WITNESS: I think an inflammatory
18 response happens on the ovarian epithelium, and some
19 ovarian cancers -- some epithelial ovarian cancers are
20 more common, serous carcinoma being the most common.
21 BY MR. ZELLERS:
22   Q. Is it your opinion that inflammation is a
23 cause of clear cell and mucinous ovarian cancer? Or
24 do you not have an opinion?
25   A. I don't have an opinion.

Page 221

1   Q. You have not done an expert review of the
2 inflammation evidence yourself, have you?
3   MS. O'DELL: Object to the form.
4   THE WITNESS: I'm aware of -- I've done
5 a review and have been aware of inflammation in
6 gynecologic cancers, especially ovarian cancer, with
7 elevated serum biomarkers suggesting inflammation and
8 also more biologic -- the laboratory work that
9 Dr. Saed and others have done.
10 BY MR. ZELLERS:
11   Q. You do know that not all inflammatory
12 conditions lead to cancer; correct?
13   A. Yes.
14   Q. There's conditions that are inflammatory
15 reactions that all of us may have -- or that folks may
16 have that don't lead to cancer, such as rheumatoid
17 arthritis; is that right?
18   A. That's, best as I understand, rheumatoid
19 arthritis.
20   Q. Same with psoriasis; is that right?
21   A. Yes.
22   Q. Those are chronic inflammatory diseases;
23 correct?
24   A. Of the skin.
25   Q. Rheumatoid arthritis is a chronic

Daniel L. Clarke-Pearson, M.D.

Page 222

1 inflammatory disease of the skin?
2   A. It can have -- in joints. There can be a
3 skin component to rheumatoid arthritis. I thought you
4 were talking about psoriasis.
5   Q. How does an acute inflammatory response lead
6 to cancer?
7   A. An acute inflammatory response, I don't
8 believe, leads to cancer.
9   Q. You have -- well, strike that.
10   On page 9 of your report, you conclude that
11 (as read):
12   "Talcum powder products is a
13   causative factor in the
14   development of epithelial ovarian
15   cancer."
16   Is that right?
17   A. Yes.
18   Q. We can change that now based upon your
19 testimony that talcum powder products is a causative
20 factor in the development of serous ovarian cancer;
21 correct?
22   MS. O'DELL: Object to the form.
23   THE WITNESS: I think I would stay with
24 epithelial ovarian cancer till we have more data.
25

Page 223

1 BY MR. ZELLERS:
2   Q. How do you define the term "talcum powder
3 products"?
4   A. Talcum powder products are Johnson's baby
5 powder and Shower to Shower.
6   Q. Are other consumer talcum powder products
7 included in your conclusions?
8   A. Yes, but Johnson & Johnson has the market
9 share, as I understand it.
10   Q. Do you understand that some of the talc
11 epidemiology separates use by type of talcum powder
12 product?
13   MS. O'DELL: Object to the form.
14   THE WITNESS: I'm not sure what you
15 mean by type of talcum powder.
16 BY MR. ZELLERS:
17   Q. Do you include talc-containing deodorizing
18 sprays in your definition of talcum powder products?
19   THE WITNESS: No. We've been talking
20 today, I thought, about Johnson -- as you defined it
21 to start the day as Johnson & Johnson baby powder and
22 Shower to Shower.
23 BY MR. ZELLERS:
24   Q. Is there a certain amount of talcum powder
25 that a product must contain to cause inflammation?

Page 224

1   A. We don't know that information.
2   Q. Do you consider cornstarch to be a talcum
3 powder product that causes inflammation?
4   MS. O'DELL: Object to the form.
5   THE WITNESS: It's not a talcum powder
6 product.
7 BY MR. ZELLERS:
8   Q. What about a product like Shower to Shower,
9 which contains cornstarch and talcum powder?
10   A. And your question is?
11   Q. My question is, is there a certain amount of
12 talcum powder that a product must contain to cause
13 inflammation?
14   A. Not that we're aware of.
15   Q. 1 percent talcum powder, 99 percent
16 cornstarch, that could cause inflammation resulting in
17 epithelial ovarian cancer. Is that your testimony?
18   A. I think that's possible.
19   Q. What methodology have you arrived -- strike
20 that.
21   What methodology have you employed to arrive
22 at the conclusion that the Shower to Shower product
23 causes inflammation?
24   A. It has talcum powder in it.
25   Q. Your opinion that talcum powder products

Page 225

1 cause inflammation is not based on the determination
2 that there is a threshold amount of talcum powder that
3 is required to be in the product before you can
4 conclude that the product will cause chronic
5 inflammation; correct?
6   MS. O'DELL: Object to the form.
7   THE WITNESS: I think there's no
8 threshold amount that -- below which the patient
9 that's exposed to talcum powder is safe.
10 BY MR. ZELLERS:
11   Q. Is there a study that you can cite me to for
12 that proposition?
13   A. No, except that, overall, women that have
14 been exposed to talcum powder in the perineum have an
15 increased risk of ovarian cancer. And we don't know
16 the quantity in each individual patient. So some
17 patients may have had a small amount and developed
18 ovarian cancer, unfortunately.
19   Q. If inflammation is the issue, why would
20 cornstarch be a superior alternative to talc?
21   A. Because I don't believe cornstarch causes
22 chronic inflammation. It's absorbed by the body.
23 Macrophages come in and clear it out. It's not a
24 permanent mineral like talc is.
25   Q. Are you aware that the FDA banned the use of

Daniel L. Clarke-Pearson, M.D.

Page 226

1 cornstarch on surgical gloves because of the risk of
2 inflammation, granulomas, fibrosis, adhesions, and
3 irritation?
4   A. Yes, because that was causing an acute
5 inflammation, not a chronic inflammation.
6   Q. Are you aware, though, that that was the
7 reason the FDA banned the use of cornstarch on
8 surgical gloves?
9   A. They were trying to stop adhesion formation
10 after surgery.
11   Q. So you are aware of that; is that right?
12   A. Yes. When I was coming up, we had to wash
13 our gloves before we operated, for that reason.
14   Q. How many patients with ovarian cancer have
15 you operated on over the course of your career?
16   A. I would say probably 30 women a year over 40
17 years.
18   Q. For those patients that had nonendometrioid
19 ovarian cancer, have you seen evidence of inflammation
20 when you operated?
21   MS. O'DELL: Object to the form.
22   THE WITNESS: When I operated,
23 75 percent of these patients have cancer all over
24 their abdominal and peritoneal cavity, and identifying
25 inflammation visually from the cancer is something a

Page 227

1 surgeon or any doctor can't do.
2   If you look at histologic specimens of the
3 tumor -- the cancer, we see inflammation, we see
4 lymphocytes and other inflammatory cells. And, in
5 addition, you see inflammatory biomarkers like CA-125.
6 BY MR. ZELLERS:
7   Q. At least grossly, when you operate on
8 patients with nonendometrioid ovarian cancer, you do
9 not see evidence of inflammation; correct?
10   MS. O'DELL: Object to the form.
11   THE WITNESS: Well, I see --
12   MS. O'DELL: I'm sorry.
13   THE WITNESS: -- probably more acute
14 inflammation. We do see additional increased
15 peritoneal fluid, what's called ascites, which is
16 probably an inflammatory response to the cancer.
17 BY MR. ZELLERS:
18   Q. Do you see adhesions?
19   A. Sometimes.
20   Q. So it's your testimony that, when you operate
21 on patients with nonendometrioid ovarian cancer, you
22 do see evidence of inflammation grossly; is that
23 right?
24   A. Yes, with ascites.
25   Q. What else?

Page 228

1   A. That's about the only thing that I can
2 determine with my naked eye as to what looks like
3 inflammation.
4   Q. You see that in some patients but not all
5 patients with ovarian cancer; correct?
6   A. That's true. That's not the only thing that
7 is related to inflammation.
8   Q. For your patients with a nonendometrioid
9 ovarian cancer, has microscopic examination of their
10 tissues shown evidence of activation of an
11 inflammatory cascade?
12   MS. O'DELL: Object to the form.
13   THE WITNESS: I don't think that
14 pathologists look at that. And I'm not sure exactly
15 what you would identify histologically in an
16 inflammatory cascade. I described to you lymphocytes,
17 for example, that represent inflammation.
18 BY MR. ZELLERS:
19   Q. Has it shown evidence of granulomas?
20   A. No.
21   MS. O'DELL: Object to the form.
22 BY MR. ZELLERS:
23   Q. Has it shown evidence of foreign body or
24 giant cell reactions?
25   A. Not that I'm aware of.

Page 229

1   Q. Do you believe that every time a talc
2 particle enters the human body, it produces an
3 inflammatory response?
4   A. A talc particle? Are we talking about platy
5 talc or fibrous talc or what kind of talc --
6   Q. Talcum powder. Do you believe that every
7 time a talc particle -- talcum powder enters the human
8 body, it produces an inflammatory response?
9   A. I think it does on a microscopic basis, yes.
10   Q. You rely on Heller 1996 for the idea that
11 talc can migrate to the ovaries. We talked about the
12 Heller paper; right?
13   A. Yes.
14   Q. And, in fact, didn't Heller find that there
15 was no reaction in the ovaries to the talc particles?
16   A. I'd like to look at that paper again --
17   Q. Sure. Take --
18   A. -- because we were talking along the lines of
19 what ovarian cancer patients look like and now we're
20 back to --
21   Q. I can get it for you or your counsel can show
22 you.
23   I'm looking at Heller 1996, page 1508, right
24 column, second-to-last paragraph.
25   Counsel, is it easier for me to find it?

Daniel L. Clarke-Pearson, M.D.

Page 230

1   MS. O'DELL: Yeah, why don't you do
2   that?
3   MR. ZELLERS: All right. We'll mark
4   the Heller paper that we discussed previously as
5   Exhibit 27.
6   (Exhibit No. 27 was marked for identification.)
7   BY MR. ZELLERS:
8   Q. Doctor, is this the paper we talked about
9   previously and that you reviewed and are relying on in
10   this case?
11   A. Yes.
12   Q. Turn, if you will, to page 1508, the second
13   page. And I'm looking on the right-hand column just
14   two sentences above "Comment" (as read):
15   "There was no evidence of response
16   to talc, such as foreign body
17   giant cell reactions or fibrosis
18   in the tissue."
19   Did I read that correctly?
20   A. Yes.
21   Q. What evidence is there that externally
22   applied talcum powder causes chronic inflammation?
23   A. Again, I think we see increased biomarkers.
24   I think Dr. Saed's research using ovarian cancer cells
25   shows the inflammatory response that results in gene

Page 231

1   mutations.
2   Q. Well, we talked a bit ago, you're unaware of
3   any reports or studies in the literature of externally
4   applied talc leading to inflammation, granulomas,
5   fibrosis, or adhesions anywhere along a woman's
6   reproductive tract; is that right?
7   MS. O'DELL: Object to the form.
8   THE WITNESS: So what you're describing
9   with adhesions is a reaction -- is an acute
10   reaction -- acute inflammatory reaction, not a chronic
11   reaction.
12   BY MR. ZELLERS:
13   Q. My question is if up to 50 percent of US
14   women have used genital talc, shouldn't this be a
15   common finding, inflammation, granulomas, fibrosis
16   along a woman's reproductive tract?
17   MS. O'DELL: Object to the form.
18   THE WITNESS: Those conditions you're
19   describing are the reaction to an acute inflammation.
20   We're talking about chronic inflammation.
21   BY MR. ZELLERS:
22   Q. So your testimony is inflammation,
23   granulomas, fibrosis, or adhesions are inconsistent
24   with and not associated with chronic inflammation in
25   your experience; is that right?

Page 232

1   MS. O'DELL: Object to the form.
2   THE WITNESS: That's correct.
3   BY MR. ZELLERS:
4   Q. In your report, you state (as read):
5   "An inflammatory reaction caused
6   by talcum powder on the tube and
7   surface of the ovary results in
8   genetic mutations and
9   carcinogenesis."
10   Is that right?
11   A. Yes.
12   Q. And you cite on page 9 in your report --
13   well, strike that.
14   So what authority supports that statement?
15   A. What was the question again?
16   Q. Sure. In your report, page 9, under
17   "Plausibility," second sentence, you state (as read):
18   "An inflammatory reaction caused
19   by talcum powder on the tube and
20   surface of the ovary results in
21   genetic mutations and
22   carcinogenesis."
23   What authority supports that statement?
24   A. The sequence of events from perineal talc
25   exposure to ovarian cancer and the mechanism of

Page 233

1   chronic inflammation on that ovary over a period of
2   time results in the gene mutation which then becomes
3   ovarian cancer.
4   Q. On what authority, on what study, are you
5   relying for that statement?
6   A. On the epidemiologic data showing that the
7   use of perineal talc results in ovarian cancer.
8   Q. But those studies don't state and find that
9   it's an inflammatory reaction caused by talcum powder
10   on the tube and the ovary, do they?
11   A. By the time the patient has ovarian cancer,
12   you don't see that.
13   Q. So my question is you've made the statement,
14   "An inflammatory reaction caused by talcum powder on
15   the tube and surface of the ovary results in genetic
16   mutations and carcinogenesis."
17   What study can I go look at, what study can
18   I read, what study are you relying on for that
19   statement?
20   A. What I just described to you. The study is
21   that the patients have ovarian cancer.
22   Q. Please name the study that you're relying on
23   for that proposition.
24   A. All the epidemiologic studies that we've been
25   talking about today in totality show the association

Daniel L. Clarke-Pearson, M.D.

Page 234

1 between the exposure of talcum powder to women's
2 perineum and ovarian cancer.
3   Q. And it's your testimony that all of those
4 studies discuss the inflammatory reaction as the
5 causal mechanism; is that right?
6   MS. O'DELL: Object to the form.
7   THE WITNESS: Those studies do not
8 discuss the mechanism in all studies. Some do.
9 BY MR. ZELLERS:
10   Q. So here's what I want: You're saying here
11 "An inflammatory reaction caused by talcum powder on
12 the tube and surface of the ovary results in genetic
13 mutations and carcinogenesis."
14   What study are you referring to, are you
15 relying on, for that statement?
16   A. That the patient got ovarian cancer. She had
17 carcinogenesis. She had gene mutations caused by
18 chronic inflammation that led to cancer. And then we
19 operated on the patient and found she had cancer.
20   Q. What is the study that says that the
21 mechanism, the biologic mechanism, was an inflammatory
22 reaction caused by talcum powder on the tube and
23 surface of the ovary?
24   A. Would you like to turn to laboratory studies?
25   Q. Is there a study that you're relying on for

Page 235

1 that statement?
2   A. There's no way somebody could do a study.
3   Q. All right.
4   A. They do serial biopsies of the ovary, watch
5 for those gene mutations, and then watch for cancer to
6 occur, and then say, hey, chronic inflammation led to
7 cancer several years later. I don't know how anybody
8 could do such a study.
9   Q. In your report, you state -- this is also on
10 page 9, under "Coherence" (as read):
11   "Epidemiologic data, in vitro and
12   in vivo research, are consistent
13   in explaining the pathogenesis of
14   epithelial ovarian cancer through
15   the inflammatory methods described
16   above."
17   Did I read that correctly?
18   A. Yes, sir.
19   Q. How does epidemiological data support your
20 inflammation theory?
21   MS. O'DELL: Objection to the form.
22   THE WITNESS: The inflammation theory
23 is the only plausible theory that I think we have to
24 explain why talcum powder can cause ovarian cancer.
25   And we see, then, in Dr. Saed's laboratory

Page 236

1 that inflammation is occurring when Johnson's baby
2 powder is put into culture with a very normal ovarian
3 cancer -- normal ovarian cells.
4 BY MR. ZELLERS:
5   Q. You'd agree that the research regarding
6 whether chronic inflammation can cause ovarian cancer
7 is ongoing; is that right?
8   A. I think cancer research in general is
9 ongoing.
10   Q. Most of the studies that you cite talking
11 about chronic inflammation refer to chronic
12 inflammation as a hypothesis of one of the ways cancer
13 might form in the ovary; is that right?
14   MS. O'DELL: Object to the form.
15   THE WITNESS: I think it's the most
16 likely -- more likely than not that's the reason that
17 ovarian cancer forms on the ovary.
18 BY MR. ZELLERS:
19   Q. But it is a hypothesis which scientists and
20 medical professionals are studying; is that right?
21   MS. O'DELL: Objection to form.
22   THE WITNESS: It's being studied, and
23 evidence coming out of laboratories is confirming that
24 hypothesis that we have in human beings.
25

Page 237

1 BY MR. ZELLERS:
2   Q. You are familiar with a paper published by
3 Merritt in 2008, "Talcum Powder, Chronic Pelvic
4 Inflammation, and NSAIDs in Relation to Risk of
5 Epithelial Ovarian Cancer"; is that right?
6   A. I've seen it.
7   Q. All right. And you cite that in Exhibit B to
8 your report. We've marked that as Exhibit 6 to this
9 deposition.
10   That's an Australian-wide case-control study
11 of around 1500 women with invasive and low malignant
12 potential ovarian tumors and 1500 population-based
13 controls.
14   Does that refresh your recollection?
15   MS. O'DELL: Are you speak of Merritt
16 2007?
17   MR. ZELLERS: I thought I was speaking
18 of Merritt 2008, which the doctor refers to in his
19 additional materials-considered list on page 17.
20   MS. O'DELL: Let's make sure we've got
21 that. And that's "Talcum Powder, Chronic
22 Inflammation, NSAIDs in Relation to the Risk of
23 Epithelial Ovarian Cancer"?
24   MR. ZELLERS: That's correct.
25   MS. O'DELL: Okay.

Daniel L. Clarke-Pearson, M.D.

Page 238

1 BY MR. ZELLERS:
2   Q. And let me try to cut to the chase, Doctor,
3 so when you look at it, we can --
4   The study concludes that, on balance,
5 chronic inflammation does not play a major role in the
6 development of ovarian cancer; is that right?
7   A. I would have to reread this study if you're
8 reading from some particular place. I don't recall
9 exactly how this study was even designed or executed.
10   Q. Take a look -- and we'll mark this as an
11 exhibit. Deposition Exhibit 28 is the Merritt paper.
12   (Exhibit No. 28 was marked for identification.)
13 BY MR. ZELLERS:
14   Q. Doctor, is this the same as what you're
15 looking at there?
16   A. Yes.
17   Q. This is a study that you cite in support of
18 your opinions; is that right?
19   MS. O'DELL: Object to the form.
20 I think it's referenced in his materials list. It's
21 not cited in his report.
22 BY MR. ZELLERS:
23   Q. It's a study that you felt was at least
24 important enough to refer to in your
25 materials-considered list; is that right?

Page 239

1   A. Along with all these other materials, yes.
2   Q. Well, if we go to the "Discussion" on
3 page 174 of Deposition Exhibit 28 -- are you with me
4 on 174?
5   A. I'm on 174. Which paragraph?
6   Q. Well, the very first --
7   A. Can I back up? I'd like to refresh my memory
8 of what this study was about.
9   It was a case-control study, 1500 patients.
10 Confirmed statistical significance of increased
11 ovarian cancer risk associated with use of talc.
12 Relative risk 1.17. Strongest were serous. I'm
13 trying to get down to your inflammation question.
14   Q. Well, it also talks about --
15   MS. O'DELL: I don't think the doctor
16 was finished.
17   MR. ZELLERS: Okay. If the doctor
18 wasn't finished, what else do you need to say, Doctor,
19 before --
20   THE WITNESS: I'm trying to find out
21 where -- all's I'm reading is the abstract, not even
22 the details of the study so far.
23 BY MR. ZELLERS:
24   Q. So I'd like you to go to "Discussion," which
25 is on page 174.

Page 240

1   A. Okay. Without knowing what -- how we got to
2 this discussion, go right ahead.
3   Q. Well, I'm citing your paper or at least one
4 of the papers you read and considered.
5   A. I have not read every word of every one of
6 these papers. And you can imagine that, and you can
7 appreciate that.
8   Q. You've not read the studies that are
9 contained in your materials-considered list --
10   MS. O'DELL: Objection.
11 BY MR. ZELLERS:
12   Q. -- Exhibit 6 to the deposition?
13   MS. O'DELL: Excuse me. Objection.
14 Misrepresents his testimony.
15   What's your question?
16 BY MR. ZELLERS:
17   Q. Well, do you want to answer that question?
18 You've not read each and every one of the studies;
19 correct?
20   MS. O'DELL: Objection. Misrepresents
21 his testimony. I think what he had testified to
22 earlier is that he had not read every word of every
23 study but had read the abstracts of -- certainly of
24 every one.
25   THE WITNESS: Right. And I haven't

Page 241

1 committed every abstract to memory. I'm sure you can
2 appreciate that too.
3 BY MR. ZELLERS:
4   Q. I can, and that's why you have it in front of
5 you.
6   A. Okay.
7   Q. So if we go to page 174, "Discussion," do you
8 see that? See that paragraph on the left-hand side?
9   A. I see the page. Which paragraph do you want
10 to see?
11   Q. Well, do you see the word "Discussion"?
12   A. Yes.
13   Q. All right. The first paragraph under
14 "Discussion," the last sentence (as read):
15   "These results, in combination
16   with previous studies, suggest
17   that chronic inflammation is
18   unlikely to play a major role in
19   the development of ovarian
20   cancer."
21   Is that the statement -- did I read that
22 correctly?
23   A. I don't think so. Says (as read):
24   "May be linked to increased risk
25   of developing ovarian cancer."

Daniel L. Clarke-Pearson, M.D.

Page 242

1   Are we reading the same -- you're reading
2 the first sentence under "Discussion"?
3   Q. No. I'm reading the last sentence of
4 "Discussion" -- last sentence of the first paragraph.
5   A. Okay. You read it correctly.
6   Q. All right. And then if we go to the
7 right-hand side, on the same page of the last
8 paragraph, the first two sentences state (as read):
9   "If inflammation plays a role in
10   the etiology of ovarian cancer,
11   then it would be expected that PID
12   would be associated with increased
13   risk of ovarian cancer. PID was
14   not associated with elevated risk
15   of ovarian tumors in our data,
16   confirming several previous
17   reports of no association with PID
18   in studies of all subtypes of
19   ovarian cancer."
20   Did I read that correctly?
21   A. You did.
22   Q. So this study concludes that, on balance,
23 chronic inflammation does not play a major role in the
24 development of ovarian cancer; correct?
25   A. So PID is pelvic inflammatory disease. Is

Page 243

1 that what you understand it?
2   Q. Yes.
3   A. So pelvic inflammatory disease is an acute
4 infection treated with antibiotics and usually
5 resolves with proper treatment. So it's not a chronic
6 infection. Having said that, PID is recognized as a
7 risk factor in many of the studies -- many of the
8 documents that you've referred to earlier this
9 morning.
10   So this particular case-control study
11 doesn't identify PID as a risk; but, in totality,
12 pelvic inflammatory disease is considered a risk
13 factor for ovarian cancer.
14   Q. What study do you rely on for your opinion
15 that pelvic inflammatory disease is a risk factor or
16 causative of ovarian cancer?
17   A. If I could turn back to the documents you
18 were using earlier today from either the CDC or --
19   Q. And just refer to them generally, and then
20 we'll take a look. The CDC --
21   A. Well, I mean, the risk -- I'm not sure which
22 one it was, but they are --
23   Q. Let me ask another question, then.
24   What methodology did you employ to consider
25 the findings of the Merritt paper in coming to the

Page 244

1 opinions contained in your report?
2   MS. O'DELL: Objection to form.
3   THE WITNESS: That it is well
4 established, in my opinion, that pelvic inflammatory
5 disease is a risk factor for ovarian cancer.
6 BY MR. ZELLERS:
7   Q. Do you agree you cannot ignore the data that
8 doesn't support your opinion and are only relying or
9 looking at data that does support your opinion?
10   MS. O'DELL: Object to the form.
11   THE WITNESS: My opinion is based on a
12 larger body of evidence and that other authorities,
13 not my opinion, have established that PID is a risk
14 factor.
15   MS. BOCKUS: Object. Nonresponsive.
16   MR. ZELLERS: Move to strike as
17 nonresponsive.
18 BY MR. ZELLERS:
19   Q. Do you agree that in doing a proper expert
20 analysis, you need to review and consider the studies
21 that both support your opinions and the studies that
22 do not support your opinions?
23   A. Absolutely. That's my methodology.
24   Q. And you believe that you have done that in
25 the discussion in your report; is that right?

Page 245

1   A. I believe so.
2   Q. All right. Do you agree that the studies
3 relating to NSAIDs are not consistent in terms of
4 establishing that NSAIDs, which reduce inflammation,
5 are associated with reduced ovarian cancer risk?
6   A. That's my understanding.
7   Q. Wouldn't you expect, if your theory of
8 inflammation is correct, that there would be
9 consistency among the NSAID studies and that they
10 would be consistently associated with reduced ovarian
11 cancer risk?
12   A. I'd have to review those studies in more
13 detail. I don't know what the doses of the NSAIDs
14 were, how chronically they were used, whether they
15 started at the time the chronic inflammation started
16 or later.
17   Q. Would you agree that the literature that you
18 cite and that you rely upon for your inflammation
19 theory cites and just shows inflammation, not chronic
20 inflammation, leading to cancer?
21   MS. O'DELL: Object to the form.
22   THE WITNESS: I'm talking about chronic
23 inflammation, to be clear.
24 BY MR. ZELLERS:
25   Q. Let's take a quick look at your report.

Page 246

1   Page 4, you cite Eberl 1948, Redic 1988, and
2 1993 NTP study of rats and mice for the proposition
3 that talcum powder is known to elicit an inflammatory
4 response in animals and humans. Is that right?
5   A. Yes.
6   Q. Those studies just show an acute inflammatory
7 response; is that right?
8   MS. O'DELL: Object to the form.
9   THE WITNESS: I don't recall that,
10 but...
11 BY MR. ZELLERS:
12   Q. Well, are you familiar with the FDA's 2014
13 response to the citizens petition which we talked
14 about earlier?
15   A. Yeah. Let me pull that out again.
16   Q. Sure. Do you have that available?
17   A. There's an exhibit here.
18   Q. I have it as Exhibit 19.
19   Do you see that -- do you have that in front
20 of you?
21   A. I have the exhibit.
22   Q. So go to page 3, where the authors talk about
23 the toxicologic findings.
24   Do you see that?
25   A. I'll get there in a second.

Page 247

1   Q. Sure.
2   Can I ask you a question?
3   A. Just give me one minute, please.
4   Okay.
5   Q. The FDA, in reviewing the toxicology findings
6 and specifically commenting on the 1993 National
7 Toxicology Program, published a study, they state --
8 and I'm reading now the last paragraph (as read):
9   "The study lacks convincing
10   scientific support because of
11   serious flaws in its design and
12   conduct, including the
13   investigators used micronized talc
14   instead of consumer-grade talc,
15   resulting in the experimental
16   protocol not being reflective of
17   human exposure conditions in terms
18   of particle size."
19   Did I read that correctly?
20   A. Well, yes. But that's taken out of context
21 to what's above here from the NTP report.
22   Q. Have you made a determination in this case
23 about the size of the particles in talcum powder
24 products?
25   A. They vary in size, from my understanding.

Page 248

1   Q. But the FDA noted -- and I'm looking at
2 page 4 -- that (as read):
3   "The investigators conceded that
4   they had problems with the aerosol
5   generation system and that the
6   study did not include positive and
7   negative dust controls."
8   Is that right?
9   A. That's what it says.
10   Q. The FDA went on to conclude that (as read):
11   "In light of these shortcomings, a
12   panel of experts at the 1994
13   ISRTP/FDA workshop declared that
14   the 1993 NTP study had no
15   relevance to human risk."
16   Did I read that correctly?
17   MS. O'DELL: Object to the form.
18   THE WITNESS: You read that correctly,
19 and this -- that study was -- that workshop was
20 convened a decade before this letter was written.
21 There was definitely more information available that
22 the FDA, once again, chose to not include or ignore.
23 BY MR. ZELLERS:
24   Q. Well, let's take a look at just a couple of
25 the studies that you refer to in your report.

Page 249

1   You cite to the Buz'Zard 2007 study; is that
2 right?
3   A. Yes.
4   Q. You rely on the Buz'Zard study to support
5 your view that talcum powder causes chronic
6 inflammation that leads to ovarian cancer. This is
7 page 4 of your report, second-to-last paragraph.
8   A. Yes. I'm trying to pull out the Buz'Zard
9 paper here.
10   Q. Do you need me to give it to you, or do you
11 have it in front of you?
12   A. I have it, sir.
13   Q. All right. So this study was conducted in a
14 nutritional lab, not a cancer lab; is that right?
15   A. Yes.
16   Q. The purpose of the study was to assess
17 whether there was a certain effect from pine bark
18 supplements; is that right?
19   A. There was an effect to neutralize the impact
20 of talcum powder.
21   Q. Did you consider the type of cells that were
22 evaluated in the Buz'Zard study?
23   And let me make it easy for you. The
24 Buz'Zard study used immortalized cells; is that right?
25 I'm looking at the second page, left column, "Cell

Daniel L. Clarke-Pearson, M.D.

Page 250

1 culture and treatment."
2   A. I'm trying to find where they talk about
3 human origin. Temperatures. Immortalized, yes.
4 Normal ovarian epithelium and normal granulosa cells.
5 It's not just generic immortalized cells.
6   Q. But the study used immortalized cells; is
7 that correct?
8   A. Immortalized ovarian cells.
9   Q. Did you investigate whether the ovarian cells
10 that they used were genetically altered?
11   A. Did I investigate whether they were
12 genetically altered?
13   Q. Yes.
14   A. I had no opportunity to investigate that
15 question.
16   Q. If the Buz'Zard study used genetically
17 altered ovarian cells that did not have the p53
18 protein, would that affect your analysis of Buz'Zard?
19   A. I would have to turn to a molecular biologist
20 to tell me what impact that might have had on the
21 impact of this study.
22   Q. Well, you yourself, as we talked about in the
23 very beginning today in one of your early
24 publications, a cell missing the p53 protein is not a
25 normal human ovarian cell; is that right?

Page 252

1 BY MR. ZELLERS:
2   Q. Saed. You were citing the Saed studies, both
3 2018, and now the Harper and Saed 2009 -- strike
4 that -- 2019 abstract; is that right?
5   A. Repeat the first one.
6   Q. Sure. You're relying, in part, for your
7 inflammation theory on Saed 2018, that chapter, and
8 the Harper and Saed 2019 abstract; is that right?
9   MS. O'DELL: Object to the form.
10   THE WITNESS: I'm relying on a paper --
11 a review paper published in Gyn Oncology in 2017. Is
12 that what you're talking about?
13 BY MR. ZELLERS:
14   Q. Well, I thought Saed that you cite in your
15 paper -- or your report -- was Saed 2018 and Harper
16 and Saed 2019.
17   Are you relying on a Saed 2017 paper as
18 well?
19   A. There's a review paper, "Updates on Oxidative
20 Stress in Pathogenesis of Ovarian Cancer" that I am
21 familiar with and is a very nice review paper
22 describing oxidative stress and gene mutation.
23   Q. Well, let me ask you a --
24   A. But there's two other abstracts here that
25 I think you're talking about.

Page 251

1   MS. O'DELL: Object to the form.
2   THE WITNESS: No, that's not what we
3 were talking about this morning in the one 1993 study
4 that I was a coauthor on. P53 mutation is what we
5 were talking about.
6 BY MR. ZELLERS:
7   Q. Right. Well, looking at the Figure 3 of the
8 Buz'Zard study 2007, "The inflammatory response does
9 not increase with increasing doses of talcum powder."
10   Is that right?
11   MS. O'DELL: Object to the form.
12   THE WITNESS: It does up to a point.
13 BY MR. ZELLERS:
14   Q. Then stops; is that right?
15   A. That's right. And then it goes down,
16 probably because the talcum powder was killing the
17 cells.
18   MR. ZELLERS: Move to strike as
19 nonresponsive.
20 BY MR. ZELLERS:
21   Q. In fact, the study shows that higher doses of
22 talcum powder are associated with lower ROS
23 generation; is that right?
24   MS. O'DELL: Object to the form.
25   THE WITNESS: That's what it says.

Page 253

1   Q. Do you know that Dr. Saed is a paid expert
2 for the plaintiffs' lawyers in this litigation?
3   A. No.
4   Q. Did you consider that fact in evaluating
5 Dr. Saed's work?
6   A. I believe he's an honest scientist and is
7 doing good scientific work.
8   Q. What is your basis for concluding that he's
9 an honest scientist?
10   A. He has a good reputation in the gynecologic
11 oncology community. He's published peer review
12 publications that have been -- undergone critical peer
13 review.
14   Q. Did Dr. Saed, in any of the publications that
15 you have reviewed -- 2017, 2018, and 2019 -- disclosed
16 that he's a paid expert for the plaintiff lawyers in
17 this litigation?
18   MS. O'DELL: Object to the form.
19   THE WITNESS: Not exactly in those
20 words.
21 BY MR. ZELLERS:
22   Q. Have you spoken with Dr. Saed?
23   A. No. I've never met him.
24   Q. Have you ever requested any information from
25 Dr. Saed?

Daniel L. Clarke-Pearson, M.D.

Page 254

1   A. No, I have not.
2   Q. The Saed study looked at immortalized cell
3 lines; is that right?
4   MS. O'DELL: Which study are you
5 referring to?
6   MR. ZELLERS: I'm referring to the 2018
7 and 2009 publications that you have referenced with
8 the doctor.
9   MS. O'DELL: You said 2009 --
10   MR. ZELLERS: I'm sorry. 2019. Excuse
11 me.
12   THE WITNESS: Just to be clear, just so
13 we know the authors, so you're talking about Fletcher
14 and Saed, the abstract?
15 BY MR. ZELLERS:
16   Q. I was referring to what you cite and
17 reference in your report, which, at least in part, is
18 Saed 2018 and Harper and Saed 2019.
19   Did you review those studies and are you
20 relying, at least in part, on those studies?
21   A. Those studies and then with the subsequent
22 full-length manuscript by Dr. Saed.
23   Q. All right. And you're aware that Dr. Saed
24 looked at immortalized cell lines; is that right?
25   A. That is about the only way to do that kind of

Page 255

1 research, is with immortalized cells.
2   Q. Are you aware that Dr. Saed has testified
3 that the cells were modified with a virus to make them
4 undergoing -- strike that -- to make them keep
5 undergoing division in vitro?
6   A. I was not aware of that, but it may be a
7 laboratory technique that's necessary to do continuous
8 studies on the same cell line.
9   Q. Are you aware that Dr. Saed testified that
10 the p53 gene was turned off in those cells?
11   MS. O'DELL: Object to the form.
12   THE WITNESS: I was not aware of his
13 testimony at all. I've not read his deposition.
14 BY MR. ZELLERS:
15   Q. What methodology did you use to apply the
16 Saed results to normal cells in actual organs?
17   MS. O'DELL: Object to the form.
18   THE WITNESS: I think this is the best
19 one can do, I presume -- I'm not a laboratory
20 scientist, but the best they can do to replicate
21 in vitro the impact of talcum powder on ovarian cells.
22 BY MR. ZELLERS:
23   Q. Can you cite any data showing that the
24 concentrations of exposure used in the Saed study are
25 the same as would be encountered in cosmetic use?

Page 256

1   MS. O'DELL: Object to the form.
2   THE WITNESS: I think we don't know how
3 much talcum powder gets to the ovary.
4 BY MR. ZELLERS:
5   Q. Can you cite any data showing that the level
6 of concentration of exposure used in the Saed study
7 has ever occurred in women with perineal talc use?
8   A. I think that's an unknown answer.
9   Q. Do you know what SNPs are, S-N-P-S?
10   A. Yes. Single-nucleotide polymorphisms.
11   Q. The Saed abstract and article looked at
12 single-nucleotide polymorphisms, or SNPs; is that
13 right?
14   A. That's correct.
15   Q. They are changes to the individual building
16 blocks of DNA; is that right?
17   A. Yes.
18   Q. SNPs can be caused by a number of agents or
19 factors; is that right?
20   A. I believe so.
21   Q. Most SNPs have no effect on health or
22 development; is that right?
23   MS. O'DELL: Object to the form.
24   THE WITNESS: Individual SNPs. So SNPs
25 do represent a gene mutation, and they do have impact

Page 257

1 on the carcinogenesis, if you will, or development of
2 cancer. Not in all cases.
3 BY MR. ZELLERS:
4   Q. What evidence do you have that the SNPs that
5 Dr. Saed observed are associated with ovarian cancer?
6   A. We see that this chronic inflammation caused
7 by talcum powder in his laboratory is creating SNPs,
8 gene mutations. Gene mutations then become cancer.
9   Q. What studies can you cite that show that
10 those SNPs have a statistically significant
11 association with ovarian cancer?
12   MS. O'DELL: Object to the form.
13   THE WITNESS: I would have to return to
14 the literature. There's a broad literature about SNPs
15 that are more than the laboratory right now. But the
16 combination of different SNPs is recognized as causing
17 cancer.
18   I don't know the specific SNPs that you're
19 referring to.
20 BY MR. ZELLERS:
21   Q. Other SNPs have no effect on health or
22 development; correct?
23   A. Some.
24   MS. O'DELL: Object to the form.
25

Daniel L. Clarke-Pearson, M.D.

Page 258

BY MR. ZELLERS:

1 BY MR. ZELLERS:

2   Q. Oxidative stress, would you agree that

3 reactive oxygen species are a normal part of cell

4 physiology?

5   A. To some degree.

6   Q. Do all substances that cause oxidative stress

7 also cause cancer?

8   A. No.

9   Q. Does the presence of oxidative stress in

10 tissue indicate that cancer will develop in that

11 tissue?

12   A. It can develop in that tissue.

13   MS. O'DELL: Excuse me, Mike. Whenever

14 you get to a breaking -- stopping point, we've been

15 going about an hour and 40 minutes, I think, something

16 like that.

17   MR. ZELLERS: Sure. Let me just finish

18 a couple of questions here.

19 BY MR. ZELLERS:

20   Q. The presence of oxidative stress in a tissue

21 may or may not indicate that cancer will develop in

22 that tissue; is that fair?

23   A. Yes, that's correct.

24   Q. If exposure to a substance causes oxidative

25 stress in a certain tissue, does that mean that the

Page 259

1 substance will cause oxidative stress in all types of

2 tissues?

3   A. Not necessarily.

4   Q. Does the body have protective mechanisms that

5 can limit tissue damage from oxidative stress?

6   A. Yes.

7   Q. What publications indicate that oxidative

8 stress is involved in the development of ovarian

9 cancer?

10   A. We're again talking about the evidence that

11 there's gene mutations being caused by oxidative

12 stress.

13   Q. Can you cite to me a publication?

14   A. That results in ovarian cancer?

15   Q. Yes.

16   A. No, I can't cite that to you. I can show you

17 the laboratory evidence that's leading to that

18 conclusion that it will happen one day.

19   MR. ZELLERS: Let's take a break.

20   THE VIDEOGRAPHER: Going off the record

21 at 3:22 p.m.

22   (Recess taken from 3:22 p.m. to 3:38 p.m.)

23   THE VIDEOGRAPHER: Back on the record

24 at 3:38 p.m.

25

Page 260

1 BY MR. ZELLERS:

2   Q. Dr. Clarke-Pearson, are you familiar with the

3 term "confounding"?

4   A. Yes.

5   Q. That's where the presence of another

6 association confuses the relationship between the

7 exposure and disease being studied; correct?

8   A. That sounds like a reasonable definition.

9   Q. For example, if you're studying the

10 association between coffee and pancreatic cancer, you

11 need to be mindful of whether cigarette smoking is

12 more common in coffee drinkers than in the rest of the

13 population; correct?

14   A. And if there's some synergism between the

15 two.

16   Q. Cigarette smoking could be a confounder in

17 that situation; is that right?

18   A. Yes.

19   Q. Because if more coffee drinkers are smokers

20 than non-coffee drinkers, an association between

21 coffee drinking and pancreatic cancer might be due to

22 the smoking, not the coffee drinking; correct?

23   MS. O'DELL: Object to the form.

24   THE WITNESS: That's where a researcher

25 would need to control for those variables.

Page 261

1 BY MR. ZELLERS:

2   Q. Confounding can distort results in

3 epidemiologic studies; is that right?

4   A. Yes.

5   Q. You agree that residual confounding is

6 possible in every observational study; correct?

7   A. I'm not sure I understand what "residual

8 confounding" is.

9   Q. Well, residual confounding is confounding

10 that remains even after you have controlled for known

11 confounders.

12   MS. O'DELL: Object to the form.

13   THE WITNESS: So let me read your

14 question.

15 BY MR. ZELLERS:

16   Q. Or I can ask it again.

17   A. Okay.

18   Q. I'll ask it again.

19   You agree that residual confounding is

20 possible in every observational study; correct?

21   MS. O'DELL: Object to the form.

22   THE WITNESS: That is possible.

23 BY MR. ZELLERS:

24   Q. You agree that it's possible that unmeasured

25 confounders may be present in every observational

Daniel L. Clarke-Pearson, M.D.

Page 262

1 study; correct?
2   MS. O'DELL: Objection to form.
3   THE WITNESS: Yes, that's possible.
4 BY MR. ZELLERS:
5   Q. It's impossible to say that all known and
6 unknown confounding factors have been controlled for
7 in any given study; is that right?
8   MS. O'DELL: Object to the form.
9   THE WITNESS: That's why we do
10 randomized control trials if possible.
11 BY MR. ZELLERS:
12   Q. Many new factors possibly involved in ovarian
13 cancer are just being published in the literature; is
14 that right?
15   MS. O'DELL: Object to the form.
16   THE WITNESS: What's being -- what
17 I was referring to as new factors are really the
18 biological mechanisms by which ovarian cancer occurs.
19 BY MR. ZELLERS:
20   Q. Well, through time, there have been different
21 factors or potential factors involved in ovarian
22 cancer; is that right?
23   MS. O'DELL: Object to the form.
24   THE WITNESS: Yes.
25

Page 263

1 BY MR. ZELLERS:
2   Q. Some of those are borne out and some are not;
3 is that right?
4   A. I'm not sure what you mean --
5   MS. O'DELL: Object to the form.
6   THE WITNESS: -- by factors aren't
7 borne out.
8 BY MR. ZELLERS:
9   Q. Well, at one point, was it thought that a
10 mumps virus was a potential viral etiology of ovarian
11 cancer?
12   A. Not that I'm aware of. When was that?
13   Q. You're not aware of that?
14   A. I'm not aware of it.
15   Q. All right. Well, how about Chlamydia
16 infection, a history of Chlamydia infection and a
17 history of weight gain during adolescence are two
18 recent examples of potentially new factors involved
19 with ovarian cancer; correct?
20   MS. O'DELL: Object to the form.
21   THE WITNESS: Well, we just finished
22 talking about pelvic inflammatory disease, and
23 Chlamydia is a pelvic inflammatory disease, so that
24 may be a specific new factor. But we already have
25 accepted PID as a risk factor.

Page 264

1   Obesity in adolescence may or may not be.
2 I'm not aware of the data on that.
3 BY MR. ZELLERS:
4   Q. Factors weren't controlled for, Chlamydia
5 infection, history of weight gain, those are factors
6 that were not controlled for -- strike that. Let me
7 be more precise.
8   A history of Chlamydia infection and a
9 history of weight gain during adolescence are two
10 recent factors that are being discussed among the
11 gynecologic oncology community; correct?
12   MS. O'DELL: Object to the form.
13   THE WITNESS: I'm not aware of the
14 obesity in adolescence. It may be.
15 BY MR. ZELLERS:
16   Q. Those factors were not controlled for in any
17 of the published talc ovarian cancer studies; correct?
18   A. That's correct.
19   Q. You rely on Terry 2013 in your report. It's
20 part of your graph on -- or your table on page 7; is
21 that right?
22   A. Yes.
23   Q. Terry 2013 did not adjust for hormone
24 replacement therapy usage; is that right?
25   A. I would have to look to see what he did and

Page 265

1 didn't adjust for.
2   Q. Is that easy for you to do?
3   A. I'm sorry?
4   Q. Is that easy for you to do?
5   A. It's buried in here under fine print, I'm
6 sure.
7   Q. Let me -- let me ask the question this way:
8 If hormone replacement therapy is a risk -- well,
9 strike that.
10   Is hormone replacement therapy a risk factor
11 for ovarian cancer?
12   A. We believe it is.
13   Q. If Terry 2013 -- and I'm asking you to assume
14 this.
15   If Terry 2013 did not account for that
16 potential confounding factor, then we wouldn't know
17 whether the odds ratio in the study would have been
18 lower if the authors had made that adjustment;
19 correct?
20   MS. O'DELL: Object to the form.
21   THE WITNESS: Or it may have been
22 higher.
23 BY MR. ZELLERS:
24   Q. We don't know; correct?
25   MS. O'DELL: Object to the form.

Daniel L. Clarke-Pearson, M.D.

Page 266

1　　　THE WITNESS: We don't know.
2　BY MR. ZELLERS:
3　　Q. Asbestos. You're, as you've told us today,
4　an expert in asbestos; is that right?
5　　A. I feel comfortable talking about asbestos.
6　　Q. You feel comfortable, as you told us and
7　testified earlier, testifying as an expert on
8　asbestos; is that right?
9　　　MS. O'DELL: Object to the form.
10　　　THE WITNESS: I don't think I said
11　I was an expert in asbestos.
12　BY MR. ZELLERS:
13　　Q. Well, on page 9 of your report, you say
14　(as read):
15　　　"There are numerous reports in the
16　　　medical literature of minerals
17　　　similar to talc causing cancer.
18　　　Probably the most significant
19　　　example is asbestos and lung
20　　　cancer/mesothelioma."
21　　Is that right?
22　　A. Yes. I'm trying to find where I say that.
23　I -- it sounds perfectly right.
24　　I'm sorry. I'm having a hard time finding
25　it. I looked under -- which topic are you reading

Page 267

1　from?
2　　Q. All right. You got page 9, under "Analogy"?
3　Or --
4　　A. Yes.
5　　Q. "There are numerous reports in the medical
6　literature of minerals similar to talc causing cancer.
7　Probably the most significant example is asbestos and
8　lung cancer/mesothelioma."
9　　Did I read that correctly --
10　　A. Yes.
11　　Q. -- from your report?
12　　A. That's correct.
13　　Q. How is talc similar to asbestos?
14　　A. First of all, the -- a number of components
15　in talcum powder have carcinogens in them. There's
16　evidence that we haven't talked about yet that
17　Johnson & Johnson baby powder and Shower to Shower had
18　asbestos in it, that fibrous talc is a carcinogen
19　according to IARC.
20　　And, in addition, heavy metals that are
21　contained in Johnson & Johnson baby powder, two of
22　them are considered carcinogens also.
23　　MR. ZELLERS: Move to strike as
24　nonresponsive.
25

Page 268

1　BY MR. ZELLERS:
2　　Q. How is talc similar to asbestos?
3　　A. Talc has fibrous talc in it. Assuming
4　there's -- let me just make an assumption that there's
5　no asbestos in talc. So that's what you're asking me
6　about.
7　　Q. I'm asking you --
8　　A. A hypothetical that talc doesn't have --
9　talcum powder doesn't have asbestos in it.
10　　Q. My question to you is that you state here
11　that there are minerals similar to talc causing
12　cancer. And what I want to know is how is talc as a
13　mineral similar to asbestos?
14　　A. Talc has a fiber in it. Fibrous talc is
15　similar to asbestos.
16　　Q. Can you be any more specific?
17　　　MS. O'DELL: Object to the form.
18　　　THE WITNESS: It's considered a
19　carcinogen. It's a long bundle of fibers.
20　BY MR. ZELLERS:
21　　Q. Talc is a long bundle of fibers?
22　　A. Fibrous talc is.
23　　Q. Well, I'm asking you about talc right now.
24　Is talc different than fibrous talc?
25　　A. If you are talking hypothetically about platy

Page 269

1　talc only --
2　　Q. I'm talking about you as an expert and
3　describing for us the differences in the minerals
4　talc, fibrous talc, and asbestos.
5　　A. So platy talc hypothetically is probably not
6　like asbestos, but it contains fibrous talc, which is
7　a long, elongated mineral that can act in the human
8　body similar to asbestos.
9　　Q. Can you be any more descriptive, or is that
10　as far as you can go in terms of explaining how
11　fibrous talc is similar to asbestos?
12　　A. Both cause a chronic inflammation in normal
13　tissues and then go on to cause oxidative stress and
14　mutations.
15　　Q. I'm talking more about the minerals. Can you
16　be any more descriptive about how fibrous talc, the
17　mineral, is similar to asbestos?
18　　　MS. O'DELL: Objection to form.
19　　　THE WITNESS: Pictures I've seen look
20　like asbestos particles, and fibrous talc looked very
21　similar.
22　BY MR. ZELLERS:
23　　Q. What other minerals that are similar to talc
24　cause cancer?
25　　　MS. O'DELL: Object to the form.

Daniel L. Clarke-Pearson, M.D.

Page 270

1   THE WITNESS: I'm not aware of any.
2 BY MR. ZELLERS:
3   Q. Are your opinions in this case dependent on
4 talcum powder containing asbestos?
5   A. No.
6   Q. Do you believe that talcum powder that does
7 not contain asbestos causes ovarian cancer?
8   A. Yes.
9   Q. If your -- if your assumption about
10 contamination of talcum powder products with asbestos
11 were not true, would that change your opinion in this
12 case?
13   A. No.
14   MS. O'DELL: Object to the form.
15 BY MR. ZELLERS:
16   Q. Is it fair to say that you have not made any
17 independent determination that the Johnson's baby
18 powder and talcum powder products are contaminated
19 with asbestos?
20   MS. O'DELL: Objection to form.
21   THE WITNESS: The only determination
22 I've had is the evidence that I've seen.
23 BY MR. ZELLERS:
24   Q. You don't have the personal expertise to make
25 that determination; is that right?

Page 271

1   A. I have the personal expertise to read reports
2 from experts and --
3   Q. Do you have the personal expertise to do the
4 testing necessary to determine whether or not talc is
5 contaminated with asbestos?
6   A. No, I do not.
7   Q. You're relying on the reports of Longo for
8 that information; is that right?
9   MS. O'DELL: Object to the form.
10   THE WITNESS: And I think also testing
11 that was performed by Johnson & Johnson, reported in
12 the John Hopkins deposition.
13 BY MR. ZELLERS:
14   Q. Well, you're talking about the two exhibits
15 that you looked at, one exhibit in John Hopkins'
16 deposition and one exhibit in Julie Pier deposition;
17 is that right?
18   A. Yes.
19   Q. You were given those documents by
20 Dr. Thompson and counsel for plaintiffs; is that
21 right?
22   A. Or by Ms. O'Dell, I'm not sure who.
23   Q. Or by Ms. O'Dell. I'll put her in the
24 counsel of plaintiffs.
25   You did not undertake a review of the

Page 272

1 literature on the topic of the alleged presence of
2 asbestos in talcum powder; is that right?
3   MS. O'DELL: Object to the form.
4   THE WITNESS: The literature?
5 BY MR. ZELLERS:
6   Q. You're relying for their -- strike that.
7   For the proposition that there is asbestos
8 in the Johnson's baby powder and Shower to Shower
9 product, your reviewing on the documents you were
10 provided by counsel, the exhibit from John Hopkins'
11 deposition, the exhibit from Julie Pier, and from the
12 selected company documents they provided to you;
13 correct?
14   A. I'm also relying on a publication by A.M.
15 Blount.
16   Q. That's what we identified earlier; is that
17 right?
18   A. I believe so.
19   Q. The A.M. Blount article deals with
20 mesothelioma, not ovarian cancer; is that right?
21   MS. O'DELL: Objection to form.
22   THE WITNESS: It talks about the
23 presence of asbestos in talcum powder.
24 BY MR. ZELLERS:
25   Q. Do you know that the deposition exhibits that

Page 273

1 you were given -- the exhibit to John Hopkins'
2 deposition and the exhibit to Julie Pier's
3 deposition -- that they were tables and exhibits that
4 were created by the plaintiff attorneys?
5   MS. O'DELL: Objection to form.
6   THE WITNESS: I'm not aware of how
7 these tables were created.
8 BY MR. ZELLERS:
9   Q. Do you know where the data in those exhibits
10 came from?
11   A. I do not.
12   Q. Are you -- strike that.
13   Have you made any effort to investigate any
14 alternative explanations for the data in those charts?
15 And I'm talking about the Hopkins and Pier deposition
16 exhibits.
17   A. No.
18   Q. If scientists with Johnson & Johnson
19 companies and Imerys scientists say that those tests
20 don't actually show asbestos, you have no expertise to
21 dispute that personally, do you?
22   MS. O'DELL: Object to the form.
23   THE WITNESS: Personally, no.
24 BY MR. ZELLERS:
25   Q. Have you looked at the evidence or been

Daniel L. Clarke-Pearson, M.D.

Page 274

1 provided with the evidence of Johnson & Johnson
2 companies and Imerys that, in fact, those tests do not
3 show asbestos?
4   MS. O'DELL: Object to the form.
5   THE WITNESS: You're referring to the
6 charts that I have?
7 BY MR. ZELLERS:
8   Q. Yes.
9   A. I'm not aware of that.
10   Q. Have you confirmed that any of the talc
11 samples mentioned in those charts, the two exhibits of
12 Hopkins deposition and Pier deposition, were actually
13 from talc that was used in body powder?
14   A. I believe the testing that was reported in
15 Hopkins was from Johnson & Johnson.
16   Q. Number one, have you confirmed that any of
17 the talc samples mentioned in those charts were
18 actually from talc that was used in body powder?
19   MS. O'DELL: Objection to form.
20   THE WITNESS: I can't confirm that.
21 BY MR. ZELLERS:
22   Q. You realize that the vast majority of talc
23 isn't used for body powder; correct?
24   MS. O'DELL: Objection to form.
25   THE WITNESS: I don't know.

Page 275

1 BY MR. ZELLERS:
2   Q. Did you consider any testing of Johnson &
3 Johnson or Imerys that found no asbestos in the talcum
4 powder?
5   A. I presume there is. The report by Dr. Longo
6 didn't show it in every single sample.
7   Q. Well, did you consider -- did you review any
8 of that testing of either Johnson & Johnson companies
9 or Imerys that found no asbestos?
10   A. I was not aware of any data on that to that
11 point.
12   Q. Were you provided that data or those test
13 results by counsel for plaintiffs?
14   A. No.
15   Q. Have you reviewed the FDA's testing of talcum
16 powder products?
17   A. You'd have to show me that evidence.
18   Q. Do you recall, sitting here, whether or not
19 you have been provided with the FDA's testing of
20 talcum powder products?
21   A. I believe I've seen it.
22   Q. Have you made any effort -- well, strike
23 that.
24   The FDA's testing, do you recall whether it
25 found asbestos or did not find asbestos?

Page 276

1   A. My recollection was, whatever technique they
2 used, they didn't find asbestos.
3   Q. Have you made any effort to quantify the
4 amount of any alleged contaminant in the Johnson's
5 baby powder products?
6   MS. O'DELL: Objection to form.
7   THE WITNESS: What contaminant are you
8 talking about?
9 BY MR. ZELLERS:
10   Q. Well, let's start with asbestos.
11   A. I haven't made any effort to quantify aside
12 from what's in the reports.
13   Q. Have you made any effort to quantify the
14 trace amounts of heavy metals that you contend are in
15 the baby powder?
16   A. I have not tried to quantitate that except
17 for what's in the reports.
18   Q. Have you attempted to quantify in any manner
19 the fragrance chemicals that you believe are contained
20 in the baby powder?
21   MS. O'DELL: Objection to form.
22   THE WITNESS: The fragrance chemicals
23 that I know are contained in the baby powder?
24 BY MR. ZELLERS:
25   Q. Well, you don't really know if any fragrance

Page 277

1 chemicals are contained in the baby powder. You have
2 reviewed some documents and materials prepared by
3 others which talk about that; right?
4   A. Yes.
5   Q. All right. Do you have an opinion on what
6 type of asbestos, if any, is in the Johnson's baby
7 powder?
8   A. Looking at the reports, there are several
9 types.
10   Q. Tell us what types you believe -- what types
11 of asbestos are found or -- strike that.
12   What types of asbestos are found in the baby
13 powder?
14   A. So this is from the Hopkins Report.
15 Tremolite. Crystalline. Some more crystalline.
16 Crystalline. Crystalline. Tremolite. Actinolite.
17 Actinolite.
18   Would you like me to go on?
19   Q. Well, you're just reading down from the
20 Hopkins, Exhibit 47; is that right?
21   A. That's correct.
22   Q. Do you know what type of asbestos is most
23 commonly associated with ovarian cancer?
24   MS. O'DELL: Object to the form.
25   THE WITNESS: I think they all are.

Daniel L. Clarke-Pearson, M.D.

Page 278

1 BY MR. ZELLERS:
2   Q. That's your belief? That all types of
3 asbestos are equally associated with ovarian cancer?
4   A. I think they're all carcinogens.
5   Q. Am I correct that, at least as you sit here,
6 you believe that all forms of asbestos are associated
7 with ovarian cancer?
8   A. There's never been a randomized trial
9 exposing women to different forms of asbestos to
10 determine whether one is more carcinogenic than the
11 other.
12   Q. So your answer is yes; is that right?
13   MS. O'DELL: Object to the form.
14   MS. BOCKUS: I was going to object to
15 his prior answer as nonresponsive.
16   THE WITNESS: Your question was, "Am
17 I correct?"
18 BY MR. ZELLERS:
19   Q. What I want to know --
20   A. Do I believe that all forms of asbestos are
21 associated with ovarian cancer? And the answer is
22 yes.
23   Q. Is there a particular type of asbestos that
24 is primarily associated with ovarian cancer?
25   MS. O'DELL: Objection. Asked and

Page 279

1 answered.
2   THE WITNESS: Not that I'm aware of.
3 BY MR. ZELLERS:
4   Q. What dose of asbestos is associated with
5 ovarian cancer?
6   A. We don't know. Possibly any dose.
7   Q. What type of ovarian cancer is asbestos
8 associated with?
9   I guess that goes back to the answer before.
10 You don't know. Is that right?
11   MS. O'DELL: Objection to form. That's
12 not what he said.
13   THE WITNESS: It's associated with
14 epithelial ovarian cancer.
15 BY MR. ZELLERS:
16   Q. Does the type of ovarian cancer vary based on
17 the type of asbestos?
18   MS. O'DELL: Objection. Asked and
19 answered.
20   THE WITNESS: I don't think anybody
21 knows that.
22 BY MR. ZELLERS:
23   Q. You've looked at studies that have explored
24 the potential link between asbestos and ovarian
25 cancer; is that right?

Page 280

1   A. Yes.
2   Q. Are you familiar with the limitations of that
3 research?
4   MS. O'DELL: Objection. Vague.
5   THE WITNESS: I'm not quite sure --
6 BY MR. ZELLERS:
7   Q. I'm sorry. Did you finish?
8   A. Yes.
9   Q. One of the papers you looked at -- and
10 I think it's contained in one of your folders -- was
11 the Reid 2011 paper. Is that right?
12   A. Yes.
13   Q. That was research on the potential
14 relationship between asbestos and ovarian cancer. One
15 of the limitations as discussed by Reid is that
16 there's a very small number of cases.
17   Is that right?
18   MS. O'DELL: Object to the form.
19   THE WITNESS: I believe so.
20 BY MR. ZELLERS:
21   Q. Is it true that most, if not all, of the
22 studies that you have reviewed with respect to
23 asbestos and ovarian cancer involve occupational
24 exposure?
25   MS. O'DELL: Object to the form.

Page 281

1   THE WITNESS: That's correct.
2 BY MR. ZELLERS:
3   Q. Did any of the nonoccupational asbestos
4 studies reach statistical significance?
5   A. No.
6   Q. Do you know how many women have been studied
7 in nonoccupational settings?
8   A. In this particular study, it looks like
9 Italian wives of asbestos factory workers would be in
10 nonindustrial settings is 1780 women.
11   Q. Are you aware of the difficulties that have
12 existed over time in distinguishing between peritoneal
13 mesothelioma and ovarian cancer?
14   A. I'm aware that there are some uncertainty in
15 some pathologic diagnoses, yes.
16   Q. Those difficulties potentially affect the
17 reliability of the studies; is that right?
18   A. Well, I think both epithelial ovarian cancer
19 and mesothelioma of the ovary or peritoneum are both
20 malignancy.
21   Q. Well, the studies have acknowledged that it's
22 difficult to distinguish between the two, between
23 peritoneal mesothelioma and ovarian cancer; is that
24 right?
25   A. Pathologically, that's correct.

Daniel L. Clarke-Pearson, M.D.

Page 282

1   Q. And the Reid study, again, makes that
2 finding. On the first page, in the right-hand column,
3 Number 2, "Difficulties with Diagnosis"; is that
4 right?
5   A. Yes.
6   Q. Have the studies addressed confounding and
7 independent risk factors?
8   MS. O'DELL: Object to the form.
9   THE WITNESS: Well, I'm certain that --
10 I would be quite confident that they didn't evaluate
11 these women, whether they had a BRCA1 or 2 mutation or
12 not, and other risk factors were not included.
13 BY MR. ZELLERS:
14   Q. Well, Camargo 2011. That's another study
15 that you put in one of your folders in preparation for
16 today; is that right?
17   A. Yeah.
18   Q. That study acknowledged an inability to
19 account for nonoccupational risk factors for ovarian
20 cancer other than age; is that right?
21   A. Yes.
22   Q. These researchers conducted a meta-analysis
23 to evaluate the association between asbestos and
24 ovarian cancer; is that right?
25   A. Yes.

Page 283

1   Q. And they acknowledge, as we spoke just a
2 moment ago, that they could not account for
3 nonoccupational risk factors for ovarian cancer other
4 than age; is that right?
5   A. I believe so.
6   Q. Also looking at Camargo, wouldn't you expect
7 to find higher rates of other cancers in women using
8 talc, like mesothelioma, if they are being exposed to
9 substantial amounts of asbestos?
10   MS. O'DELL: Object to the form.
11   THE WITNESS: They would be -- they
12 would have to inhale it to a quantity enough to cause
13 mesothelioma of the lung.
14 BY MR. ZELLERS:
15   Q. Are women who use talc in the perineal region
16 at greater risk of mesothelioma?
17   A. Not that I'm aware of.
18   Q. Are women who use talc in the perineal region
19 at greater risk of asbestosis?
20   A. Not that I'm aware of.
21   Q. If there was more asbestos in talcum powders
22 in the 1970s, shouldn't we have seen higher rates of
23 ovarian cancer in the earlier studies?
24   MS. O'DELL: Object to the form.
25   THE WITNESS: I think getting back to

Page 284

1 your point about confounding issues, the risk factors
2 in the 1970s above and beyond exposure to talc were
3 not always controlled for. I think we know more about
4 that today in ongoing studies.
5 BY MR. ZELLERS:
6   Q. You'd agree that exposure to asbestos through
7 the perineal cosmetic talc use, assuming that talc
8 contains asbestos fibers, is different from the heavy
9 occupational exposure that's primarily been
10 researched; is that right?
11   MS. O'DELL: Object to the form.
12   THE WITNESS: Yes, I would agree with
13 that.
14 BY MR. ZELLERS:
15   Q. Is the asbestos that women are exposed to
16 from using cosmetic talc qualitatively the same as the
17 raw asbestos encountered at a factory, if you know?
18   MS. O'DELL: Object to the form.
19   THE WITNESS: The raw asbestos
20 encountered at a factory before it's processed?
21 BY MR. ZELLERS:
22   Q. Yes.
23   A. I don't know the answer to that.
24   Q. Do you know what a cleavage fragment is?
25   A. It's part of platy talc.

Page 285

1   Q. Do you know how a cleavage fragment differs
2 from an asbestos fiber?
3   A. It has to do with the size of the fiber.
4   Q. Do you have any opinions about cleavage
5 fragments in this case?
6   A. What case are we talking about?
7   Q. You serving as an expert witness in the --
8   A. I guess I think of a case as a patient.
9   Q. Well, you're here today talking generally
10 about the risk of ovarian cancer from talcum powder
11 use; is that right?
12   A. Yes.
13   Q. Do you intend to express any expert opinions
14 in this matter about cleavage fragments?
15   MS. O'DELL: Objection to form.
16   THE WITNESS: If asked.
17 BY MR. ZELLERS:
18   Q. Okay. What opinions do you have about
19 cleavage fragments? And, specifically, how does a
20 cleavage fragment differ from an asbestos fiber?
21   A. So it has to do with the ratio of length to
22 width, and a cleavage factor has a less than 6:1
23 proportion.
24   Q. Anything else?
25   A. You were asking about cleavage fragments?

Daniel L. Clarke-Pearson, M.D.

Page 286

1   Q. Yes. And I'm asking how it differs from an
2 asbestos fiber --
3   A. Asbestos needle is longer. It's either a
4 ratio of 6:1 up to less than 15:1.
5   Q. Anything else?
6   A. And then fibers are considered greater than
7 15:1 ratio.
8   Q. Asbestos fibers or cleavage fragments?
9   A. Asbestos fibers.
10   Q. How does a cleavage fragment differ from
11 fibrous talc?
12   A. I'm not sure I know the difference.
13   Q. Does it make a difference to your theory and
14 your opinions if it turns out that talc contains
15 cleavage fragments of nonasbestiform amphiboles
16 instead of asbestiform amphiboles?
17   MS. O'DELL: Objection.
18   THE WITNESS: I'm going to have to read
19 your question.
20 BY MR. ZELLERS:
21   Q. Sure. And if you don't have opinions, that's
22 okay. I'm just trying to find out what you have
23 opinions about.
24   A. No, I don't have an opinion.
25   Q. You don't have opinions about whether or not

Page 287

1 regulatory action in this area rejects the idea that
2 science has established that cleavage fragments or
3 nonasbestiform amphiboles pose the same risk as
4 asbestos; correct? You leave that to other experts to
5 address?
6   A. The regulatory portion, yes.
7   Q. How, if at all, did you factor the difference
8 between asbestiform and nonasbestiform minerals into
9 your analysis of the relationship between talcum
10 powder use and ovarian cancer?
11   MS. O'DELL: Objection to the form.
12 Compound.
13   You may answer the question if you
14 understand it.
15   THE WITNESS: Well, I'm quite certain,
16 based on IARC, that asbestiform minerals are
17 carcinogenic.
18 BY MR. ZELLERS:
19   Q. That is your answer to my question?
20   A. Yes.
21   Q. All right. Fragrance chemicals and heavy
22 metals, you're aware those are addressed in
23 Dr. Crowley's report; is that right?
24   MS. O'DELL: Objection. Form.
25   THE WITNESS: Yes. I don't have that

Page 288

1 in front of me, though.
2 BY MR. ZELLERS:
3   Q. You're not expressing opinions in this case
4 on fragrance chemicals and heavy metals and any
5 association fragrance chemicals and heavy metals may
6 have on ovarian cancer; correct?
7   MS. O'DELL: Objection. Form.
8   THE WITNESS: No. I am expressing an
9 opinion about that.
10 BY MR. ZELLERS:
11   Q. What research have you done with respect to
12 the fragrance chemical and trace amounts of heavy
13 metals that are contained in the talcum powder?
14   MS. O'DELL: Objection to the form.
15 Compound.
16   THE WITNESS: It's my opinion that
17 talcum powder causes ovarian cancer, that talcum
18 powder contains platy talc, fibrous talc, asbestos,
19 heavy metals -- three of them -- and fragrances.
20   I'm not necessarily saying one of that list
21 is causing the cancer. It's the talcum powder -- the
22 baby talc -- baby powder and the Shower to Shower --
23 that's causing the ovarian cancer.
24 BY MR. ZELLERS:
25   Q. I understand that, and I think I've asked you

Page 289

1 my questions with respect to that.
2   What I'm asking about now is whether or not
3 you have made a separate analysis as to whether one or
4 more of the fragrance chemicals or one or more of the
5 trace heavy metals that have been reported to be
6 contained in talcum powder, whether those are causally
7 associated or a causal factor for ovarian cancer?
8   A. In combination with the commercial product
9 called baby powder and Shower to Shower, I think they
10 all contribute to the outcome, which is ovarian
11 cancer.
12   Q. Are you relying on any scientific literature
13 to support your opinion that some of the chemicals in
14 Johnson's baby powder cause ovarian cancer?
15   MS. O'DELL: Object to the form.
16   THE WITNESS: We know that they can be
17 carcinogenic.
18 BY MR. ZELLERS:
19   Q. With respect to ovarian cancer.
20   A. Not specifically to ovarian cancer. We
21 haven't studied that.
22   Q. Do you have any evidence that the fragrance
23 chemicals and trace heavy metals contained in
24 Johnson's baby powder have been tested in human beings
25 and found to cause inflammation?

Daniel L. Clarke-Pearson, M.D.

Page 290

1   A. I'm not aware of those studies.

2   Q. Is there any epidemiology, human studies,

3 substantiating the theory that fragrance ingredients

4 can cause ovarian cancer?

5   A. Fragrance ingredients by themselves?

6   Q. Yes.

7   A. I'm not aware of any study that's evaluated

8 that.

9   Q. Is there any epidemiology study

10 substantiating the theory that fibrous talc is

11 carcinogenic?

12   A. IARC claims it is carcinogenic.

13   Q. That it causes ovarian cancer, specifically?

14   A. I believe so.

15   Q. You'd defer to IARC on that; is that right?

16   MS. O'DELL: Object to the form.

17   THE WITNESS: Yes.

18 BY MR. ZELLERS:

19   Q. Is there any epidemiology substantiating the

20 theory that exposures to trace amounts of heavy

21 metals, allegedly, or that you believe are contained

22 in the Johnson's baby powder can cause ovarian cancer?

23   A. I'm not aware that anybody's done a

24 randomized trial in human beings with carcinogen --

25 carcinogenic heavy metals to evaluate whether ovarian

Page 291

1 cancer or any other cancer might occur.

2   Q. Well, aside from a randomized clinical trial,

3 are you aware of any other epidemiology substantiating

4 the theory that exposures to trace amounts of the

5 heavy metals that are reported to be in the Johnson's

6 baby powder can cause ovarian cancer?

7   MS. O'DELL: Object to the form.

8   THE WITNESS: I don't think that

9 anybody's ever studied that as a separate entity of

10 metals only exposed to the ovary.

11 BY MR. ZELLERS:

12   Q. You have no evidence that the blood or tissue

13 levels of any trace heavy metals are higher in genital

14 talc users as compared to nonusers; is that right?

15   A. That's correct.

16   Q. Are your opinions in this case depending on

17 talc containing carcinogenetic [sic] metals?

18   A. Not necessarily.

19   Q. Are your opinions in this case dependent on

20 talc containing carcinogenetic [sic] fragrances?

21   A. Not necessarily.

22   Q. Do you have any opinions or knowledge as to

23 the concentration of each of the fragrance chemicals

24 that are contained in Johnson's baby powder?

25   A. No.

Page 292

1   Q. Or Shower to Shower?

2   A. No.

3   Q. You've not done any independent testing of

4 that; correct?

5   A. That's correct.

6   Q. How, if at all, did you factor the dose

7 fragrances and heavy -- or trace heavy metals into

8 your analysis of the potential relationship between

9 those compounds and ovarian cancer?

10   A. I didn't factor in.

11   Q. Let me ask you a couple of questions about

12 the Health Canada assessment and the Taher article.

13 Those are new materials that you reviewed between the

14 time of your report and appearing here today; is that

15 right?

16   A. That's correct.

17   Q. Have you read the draft Health Canada risk

18 assessment -- I'll provide you with a copy so we know

19 what we're speaking of.

20   (Exhibit No. 29 was marked for identification.)

21   MR. ZELLERS: Deposition Exhibit 29 is

22 the draft Health Canada decision framework -- strike

23 that.

24   Exhibit 29 is the Health Canada

25 Decision-Making Framework for Identifying, Assessing,

Page 293

1 and Managing Health Risks.

2   Is that not what he's reviewed?

3   MS. O'DELL: If you're handing him that

4 and suggesting, that's not the health assessment that

5 he's reviewed.

6   MR. ZELLERS: So do we have the health

7 assessment here? And, if not, we can just identify

8 it. But I do want to ask him a few questions about

9 the --

10   MS. O'DELL: I do think we have it

11 here. But, if you're going to ask him questions,

12 I would put it in front of him. So, if we don't have

13 a hard copy, I'm happy to put my electronic copy in

14 front of him.

15   MR. ZELLERS: Well, please put whatever

16 you think you need to put in front of the witness so

17 he can answer a couple of questions about the Health

18 Canada risk assessment.

19   MS. O'DELL: Sure. Give me just a

20 moment --

21   MR. ZELLERS: Sure.

22   MS. O'DELL: -- because the copy I have

23 is marked up, and I know you prefer for me not to hand

24 him my marked-up copy.

25   MR. ZELLERS: I would prefer that.

Daniel L. Clarke-Pearson, M.D.

Page 294

1   MS. O'DELL: Doctor, if you want to
2 just use my computer, feel free to --
3   THE WITNESS: Okay. I'm not real fast
4 at running through a computer, but --
5 BY MR. ZELLERS:
6   Q. Hopefully, my questions will be pretty
7 high-level.
8   You have in front of you the draft Health
9 Canada risk assessment; is that right?
10   A. On my tablet, yes.
11   Q. Have you looked into what other public health
12 authorities have had to say about talc and ovarian
13 cancer?
14   A. Except for what the FDA has had to say.
15   Q. The answer is, no, other than with respect to
16 what the FDA has said; is that right?
17   A. The answer is no.
18   Q. Why would you rely on Health Canada but not
19 other public health organizations?
20   MS. O'DELL: Object to the form.
21   THE WITNESS: It's my understanding
22 that this is very recent analysis of the issues
23 regarding talcum powder and ovarian cancer and other
24 harms.
25

Page 295

1 BY MR. ZELLERS:
2   Q. You understand it's a draft assessment; is
3 that right?
4   A. That's correct.
5   Q. You understand that we're at the very
6 beginning of the public comment period; is that right?
7   MS. O'DELL: Object to the form.
8   THE WITNESS: I don't know that.
9 BY MR. ZELLERS:
10   Q. Are you aware that Health Canada can take up
11 to two years to take any action or no action at all?
12   MS. O'DELL: Object to the form.
13   THE WITNESS: I was not aware.
14 BY MR. ZELLERS:
15   Q. How did you come to learn of the Health
16 Canada risk assessment?
17   A. It was brought to my attention by counsel.
18   Q. By counsel for plaintiffs; is that right?
19   A. That's correct.
20   Q. Were you involved in the risk assessment
21 prior to its publication?
22   A. Was I involved?
23   Q. Yes.
24   A. No.
25   Q. Have you submitted any comments to Health

Page 296

1 Canada?
2   A. I wasn't aware -- as I said, I wasn't aware
3 that there were comments that could be made.
4   Q. Outside of your litigation consulting work,
5 do you generally rely on draft assessments by
6 regulatory agencies?
7   MS. O'DELL: Object to the form.
8   THE WITNESS: I think it's something
9 that's worth looking at. It doesn't necessarily sway
10 my opinion, but could be useful additional information
11 that might be cutting edge.
12 BY MR. ZELLERS:
13   Q. You don't cite or -- strike that.
14   You do not rely on draft regulatory
15 assessments in your peer-reviewed publications and
16 studies; is that right?
17   MS. O'DELL: Object to the form. Asked
18 and answered.
19   THE WITNESS: Not usually, but don't
20 know what -- there's information there. If there's
21 information I can extract from a draft of something
22 that's useful, I can use it.
23 BY MR. ZELLERS:
24   Q. Are you familiar with the precautionary
25 principle?

Page 297

1   A. Slightly.
2   Q. Basically, that means taking a precautionary
3 approach to decision-making that emphasizes the need
4 to take timely preventative action even in the absence
5 of a full scientific demonstration of cause and
6 effect.
7   Does that sound right?
8   A. Sounds very reasonable, yeah.
9   Q. You understand that Health Canada may have
10 made recommendations that are purely precautionary; is
11 that right?
12   MS. O'DELL: Object to the form.
13   THE WITNESS: That's what I've read,
14 yes.
15 BY MR. ZELLERS:
16   Q. I can go through the document for it if need
17 be, but in the -- its publication -- I'll hand it to
18 you -- which we've marked as Exhibit 29, it is
19 captioned "Health Canada Decision-Making Framework for
20 Identifying, Assessing, and Managing Health Risks."
21   Do you have that in front of you?
22   A. You've handed it to me, yes.
23   Q. If you go to page 5, Health Canada sets out
24 the bases for its risk assessments; is that right?
25   A. Let me get to page 5 here.

Daniel L. Clarke-Pearson, M.D.

Page 298

1   Q. Sure.
2   A. In the black box "Underlying Principles"?
3   Q. Yes, "Underlying Principles."
4   One of the underlying principles is "use a
5 precautionary approach"; is that right?
6   A. That's what it says.
7   Q. If you go, then, to page 8, second paragraph,
8 second sentence, where Health Canada sets forth "use
9 of a precautionary approach," the second sentence
10 reads (as read):
11   "A precautionary approach to
12   decision-making emphasizes the
13   need to take timely and
14   appropriately preventative action
15   even in the absence of a full
16   scientific demonstration of cause
17   and effect."
18   Did I read that correctly?
19   A. Yes, sir.
20   Q. So a recommendation by Health Canada does not
21 require a finding of causation like is required in a
22 court. Does that sound right based upon what we have
23 reviewed here?
24   MS. O'DELL: Object to the form.
25   THE WITNESS: I'm not sure what the

Page 299

1 requirements are for court. I understand the
2 precautionary portion here.
3 BY MR. ZELLERS:
4   Q. And you also understand that, with the use of
5 a precautionary approach, that action can be taken
6 even in the absence of a full scientific demonstration
7 of cause and effect?
8   MS. O'DELL: Objection to form.
9   THE WITNESS: What action are you
10 talking about?
11 BY MR. ZELLERS:
12   Q. Well, decision-making, any sort of
13 assessment.
14   MS. O'DELL: Objection to form.
15   THE WITNESS: I'm still not
16 understanding.
17 BY MR. ZELLERS:
18   Q. Sure. Health Canada --
19   A. Yes.
20   Q. -- does not need, in terms of its risk
21 assessment, to have a full scientific demonstration of
22 cause and effect?
23   A. I understand.
24   MS. O'DELL: Objection to form.
25

Page 300

1 BY MR. ZELLERS:
2   Q. All right. Thayer 2018, that's a new and
3 additional meta-analysis that you have reviewed?
4   A. Yes.
5   Q. Let's mark Thayer 2018 as Deposition
6 Exhibit 30.
7   (Exhibit No. 30 was marked for identification.)
8 BY MR. ZELLERS:
9   Q. And you can tell us if this is --
10   A. I've got a copy.
11   Q. Well, take, if you will, the court --
12 deposition exhibit number. Just put it in your pile
13 there so we can make sure we all understand what we're
14 talking about.
15   You have seen this review before; is that
16 right?
17   A. Yes, I have.
18   Q. The Health Canada risk assessment that you
19 looked at a few moments ago relies on this
20 meta-analysis by Thayer and others; is that right?
21   A. That's my understanding. They may use other
22 information too.
23   Q. Do you know whether or not Thayer 2018 has
24 been peer-reviewed?
25   A. I'm not aware of that.

Page 301

1   Q. Do you know if it has been submitted for
2 publication?
3   A. I do not know.
4   Q. How can you rely on the Health Canada risk
5 assessment without assessing the quality of one of the
6 major studies on which they rely?
7   MS. O'DELL: Objection to form.
8   THE WITNESS: And the major study
9 you're referring to is Thayer?
10 BY MR. ZELLERS:
11   Q. Yes.
12   A. Let me read the first part of your question
13 here.
14   So I'm not saying that I rely on the Health
15 Canada risk for my total opinion. It's another piece
16 of evidence and information that's helpful in me
17 coming to my opinion. And this only supports my
18 opinion.
19   Bradford Hill's breakdown is very similar to
20 my opinion. I didn't see this before I created my
21 opinion.
22   Q. Do you know if Thayer 2018 employed a
23 reliable methodology?
24   A. I believe it's very similar to other
25 methodology and systematic reviews and meta-analyses.

Daniel L. Clarke-Pearson, M.D.

Page 302

1   Q. Did you have access to the appendices or
2 supplemental tables referenced in the Thayer
3 meta-analysis?
4   A. I did not.
5   Q. Do you know the source of funding for Thayer
6 2018 meta-analysis?
7   A. If it was listed on here, I should have
8 picked it up. If not, then I don't know the answer to
9 your question.
10   Q. Do you know the credentials of the authors of
11 Thayer 2018?
12   A. None other than what are listed on the cover
13 sheet of this paper.
14   Q. Do you personally know any of the authors of
15 Thayer 2018?
16   A. No, sir.
17   Q. Do you know whether or not any of those
18 authors have conflicts of interest or potential
19 conflicts of interest?
20   A. Do not know.
21   Q. In Thayer 2018, the authors concluded that
22 "The evidence suggests that asbestos contamination
23 does not explain the positive association between
24 perineal use of talc powder and ovarian cancer."
25   Is that right?

Page 303

1   MS. O'DELL: Mike, what page are you
2 reading from?
3   MR. ZELLERS: Page 41, last sentence.
4 So we're on Deposition Exhibit 30, the Thayer
5 meta-analysis, page 41, last part.
6   MS. O'DELL: Thank you.
7 BY MR. ZELLERS:
8   Q. Doctor, I really just have a really simple
9 question.
10   A. Okay.
11   Q. So the authors conclude -- or state that
12 (as read):
13   "The similarity of findings
14   between studies published prior to
15   and after this point suggest
16   asbestos contamination does not
17   explain the positive association
18   between perineal use of talc
19   powder and risk of ovarian
20   cancer."
21   Is that right?
22   MS. O'DELL: Object to the form.
23   THE WITNESS: That's what they say.
24 BY MR. ZELLERS:
25   Q. Do you disagree with the authors on that

Page 304

1 point?
2   A. I do not disagree with the author on that
3 point.
4   Q. One of the Bradford Hill criteria that we've
5 discussed is consistency; is that right?
6   A. Yes.
7   Q. Look at Thayer 2018. So Exhibit 30, page 25,
8 Table 2.
9   Do you have that?
10   A. Yes.
11   Q. Table 2 is entitled "Summary of Evidence for
12 Each of the Hill Criteria of Causation as Applied to
13 Perineal Application of Talc and Ovarian Cancer."
14   Is that right?
15   A. I'm sorry. What were you reading -- where
16 were you reading from?
17   Q. Sure. Table 2 on page 25 --
18   A. Right.
19   Q. -- is captioned "Summary of Evidence for Each
20 of the Hill Criteria of Causation as Applied to
21 Perineal Application of Talc and Ovarian Cancer."
22   A. Yes.
23   Q. And they kind of go through the same Bradford
24 Hill factors that you do; is that right?
25   A. Yes.

Page 305

1   Q. Under "Consistency," they said that
2 (as read):
3   "15 out of 30 studies reported
4   positive and significant
5   associations."
6   Is that right?
7   A. That's right.
8   Q. We're back to, similar with Langseth, half
9 the studies showing significant associations and half
10 the studies don't. Thayer reports that same findings
11 here; is that right?
12   A. Yes, but not all studies have the same
13 weight.
14   Q. And we've discussed that before; is that
15 right?
16   A. Yes. I just wanted to bring it up again,
17 since we're talking about that topic.
18   Q. Let's go to "no dose response." And that was
19 your -- well, let me withdraw that statement.
20   Go to page 21, if you will, second
21 paragraph, last few sentences.
22   Do you have that?
23   MS. O'DELL: What page are you on?
24   MR. ZELLERS: Page 21.
25

Daniel L. Clarke-Pearson, M.D.

Page 306

BY MR. ZELLERS:

2   Q. The authors here in this section are

3 discussing whether or not there is a dose response and

4 dose response findings in the studies; is that right?

5   A. Yes.

6   Q. They conclude at the very end -- and I'm

7 looking on page 21, the last sentence above 3.3.2

8 (as read):

9   "When conducted, findings from

10   trend analyses were not

11   consistent."

12   Do you see that?

13   A. Yes, I do.

14   Q. The authors recognize that there's no

15 consistent dose response across studies, and you agree

16 with that; is that right?

17   MS. O'DELL: Objection to form.

18   THE WITNESS: I think there's some

19 evidence there's dose response. Some studies don't do

20 enough to evaluate for dose response, especially the

21 cohort studies that are pretty well destroyed back on

22 page 43.

23 BY MR. ZELLERS:

24   Q. Some studies find dose response and some

25 studies don't; correct?

Page 307

1   MS. O'DELL: Objection to form.

2   THE WITNESS: That's correct.

3 BY MR. ZELLERS

4   Q. And that's true of case-control studies; is

5 that right?

6   A. Yes.

7   Q. I want to go back to a question I had asked

8 you earlier.

9   When you do surgery and you see

10 inflammation, would you agree that inflammation that

11 you see is likely related to the cancer itself?

12   A. So let me clarify so we don't get confused.

13   The inflammation that I see is purely

14 ascites. The rest -- which is fluid in the abdomen

15 either caused by the cancer or by inflammation.

16   Q. The ascites can be caused by the cancer

17 itself; correct?

18   A. Yes.

19   MR. ZELLERS: I have no further

20 questions. Some of my colleagues may have questions

21 for you. Thank you for your time.

22   THE WITNESS: Thank you.

23   MS. BOCKUS: Could we take a quick

24 break so that we can change places?

25   MS. O'DELL: Sure.

Page 308

1   THE VIDEOGRAPHER: Going off the record

2 at 4:36 p m

3   (Recess taken from 4:36 p m to 4:44 p m)

4   THE VIDEOGRAPHER: Back on the record

5 at 4:44 p m

6 CROSS-EXAMINATION BY COUNSEL FOR THE DEFENDANT IMERYS

7 BY MS BOCKUS:

8   Q Doctor, I just want to be sure that what we

9 have marked so far will provide us with copies of all

10 of your handwritten notes

11   A Certainly

12   Q Okay Are there some handwritten notes that

13 are not on the table in front of you right now?

14   A Yeah There's some in these files and

15 some -- like this, with sticky notes

16   Q And that's what I'm looking for I want to

17 make sure I get all your sticky notes and all of the

18 notations that you have made in your review of the

19 articles

20   And so when we get -- it looks like there

21 are two binders that have flags and that sort of thing

22 in them Are there notes in the binders that are over

23 on the table?

24   A No, ma'am

25   Q Okay So other than the binders and the

Page 309

1 materials that are on the table, do you have

2 handwritten notes somewhere else?

3   A. No.

4   Q. Earlier today, you were asked a question --

5 I think it was about the FDA letter -- and you thought

6 you had some handwritten notes on that. Do you know

7 where those might be?

8   A. I don't recall now. You know, it was a

9 sticky note. Just what I've been trying to do is

10 abstract these papers to a few facts that I think are

11 important. It's not personal opinions or other things

12 like that; it's just trying to move the conversation

13 along.

14   Q. Would you agree that in general ovarian

15 cancer is a disease of aging?

16   MS. O'DELL: Objection to form.

17   THE WITNESS: That is one of the risk

18 factors, yes.

19 BY MS. BOCKUS:

20   Q. That very few women are diagnosed with

21 ovarian cancer who are under 30 years of age; correct?

22   A. With epithelial ovarian cancer, yes.

23   Q. And that risk -- so the numbers are different

24 depending which type of ovarian cancer you're talking

25 about; correct?

Daniel L. Clarke-Pearson, M.D.

Page 310

1   A. Yes.
2   Q. So confining it to epithelial ovarian cancer,
3 that risk starts to rise in the 30s and rises even
4 more in the 40s, 50s, and 60s; correct?
5   A. Yes, that's my understanding.
6   Q. And in the 60s, it kind of levels off --
7   A. In the 60s or 70s. I've forgotten what the
8 curves look like exactly.
9   Q. And other than being female of a certain age,
10 most patients who you see, you don't have any idea of
11 what caused their ovarian cancer; correct?
12   MS. O'DELL: Object to the form.
13   THE WITNESS: Again, I get back to my
14 theme about gene mutation. Something caused the gene
15 mutation to cause that normal cell that's mutated now
16 to become malignant.
17 BY MS. BOCKUS:
18   Q. Exactly. Somewhere along the aging process,
19 perhaps, or through some exposure, there's been a gene
20 mutation and -- well, let me stop there. Scratch all
21 that.
22   It actually takes multiple gene mutations
23 for a cancer to begin, does it not?
24   A. That's our understanding.
25   Q. Our understanding is that several things

Page 311

1 happen -- have to happen before a cancer cell is
2 formed; correct?
3   A. That's our usual understanding of what the
4 onset of cancer is.
5   Q. And our general understanding is that it
6 takes decades for that to happen, generally speaking;
7 correct?
8   A. It depends upon what the mutations are. A
9 woman that's born with a genetic mutation of BRCA1,
10 for example, already has some mutations. So that's
11 why we believe they develop ovarian cancer at an
12 earlier age. Just a couple more mutations, and then
13 the ovarian cancer starts.
14   Whereas a woman that doesn't have a BRCA1
15 mutation, as she gets older, she obtains or gets
16 mutations over time. And the longer you live, the
17 more likely you are to have those mutations to become
18 ovarian cancer.
19   Q. And one of the things that happens over time
20 is our body's ability to fight off detected mutations
21 decreases; correct?
22   A. Yes, in general.
23   Q. So back to my prior question, when patients
24 come to you who have ovarian cancer, other than being
25 female and over a certain age, are you ever able to

Page 312

1 tell them what caused the genetic mutation that caused
2 their cancer?
3   MS. O'DELL: Object to the form.
4   THE WITNESS: Aside from the inherited
5 BRCA mutations and Lynch syndrome, in general, no, we
6 can't.
7 BY MS. BOCKUS:
8   Q. Would you agree that what we know today about
9 what causes ovarian cancer is actually dwarfed by what
10 we don't yet know about the cause of ovarian cancer?
11   MS. O'DELL: Object to form.
12   THE WITNESS: I think it's fair to say
13 we know some risk factors.
14 BY MS. BOCKUS:
15   Q. But we're learning new risk factors and new
16 genetic mutations all the time; correct?
17   MS. O'DELL: Object to the form.
18   THE WITNESS: In general, we're moving
19 along those lines in research.
20 BY MS. BOCKUS:
21   Q. I just want to be clear. Is it your position
22 that being powdered as an infant with talc increases
23 that person's risk of being diagnosed with ovarian
24 cancer as a woman?
25   A. I think it's the sustained exposure more than

Page 313

1 if an infant was just -- received talcum powder and
2 then never continued to use it into her 20s, 30s, 40s,
3 and 50s, my opinion would be that infant is not at
4 particularly high risk.
5   Q. Is it your opinion that powdering one's baby
6 with talcum powder increases the mother's risk of
7 ovarian cancer?
8   MS. O'DELL: Object to the form.
9   THE WITNESS: So just -- just through
10 inhaled? I believe that there's not enough evidence
11 to say that.
12 BY MS. BOCKUS:
13   Q. Okay. And so fair to say that you're truly
14 confining your opinion to the theory that talc can
15 travel from the perineum to the ovary and cause
16 ovarian cancer that way; is that correct?
17   A. And cause --
18   MS. O'DELL: Object to the form.
19   Excuse me.
20   THE WITNESS: -- cause chronic
21 irritation and inflammation, yes.
22 BY MS. BOCKUS:
23   Q. In order for a cancer to be called a cancer,
24 it has to evolve in such a way that it has limitless
25 replicative potential; correct?

Daniel L. Clarke-Pearson, M.D.

Page 314

1   MS. O'DELL: Object to the form.
2   THE WITNESS: I think cancers -- if
3 I understand what you're saying, some cancers also
4 replicate rapidly and then slow down and may be
5 indolent for a period of time.
6   So the timeline of onset of cancer to death,
7 which is, I guess, the timeline, can vary from one
8 patient to another.
9 BY MS. BOCKUS:
10   Q. Cancer needs to develop the ability to evade
11 apoptosis; correct?
12   A. I'm sorry?
13   Q. Evade apoptosis.
14   A. Yeah, that's sort of -- by definition, cancer
15 has already evaded apoptosis.
16   Q. Exactly.
17   Cancer also needs to develop sustained
18 angiogenesis; correct?
19   A. It needs to derive a blood supply, and
20 angiogenesis is the blood supply.
21   Q. It needs the ability to invade other tissue
22 and metastasize; correct?
23   MS. O'DELL: Object to the form.
24   THE WITNESS: I'm not sure it needs to.
25 I mean, in general, the time course is one of invasion

Page 315

1 or metastasis or both.
2 BY MS. BOCKUS:
3   Q. Okay. Which of those steps do you believe
4 talc contributes to?
5   MS. O'DELL: Objection to form.
6   THE WITNESS: I believe talc
7 contributes to the first onset -- or the additional or
8 first onset of mutations that then lead on to cancer.
9 BY MS. BOCKUS:
10   Q. What -- in what gene does the mutation occur
11 in that talc impacts?
12   MS. O'DELL: Object to the form.
13   THE WITNESS: Some genes -- SNPs that
14 Dr. Saed has identified are what we know, I think, to
15 date. We know there's other genetic mutations that
16 are present in the somatic form of ovarian cancer as
17 well as the inherited genes.
18   But I don't think anybody has studied that
19 in correlation with talc exposure, so that would be an
20 interesting investigation to undertake.
21 BY MS. BOCKUS:
22   Q. Inflammation -- chronic inflammation, is that
23 associated with pain?
24   A. With pain?
25   Q. Yes.

Page 316

1   A. It might be.
2   Q. Is chronic inflammation associated -- well,
3 let me back up.
4   You testified earlier that you would not
5 expect to see signs of chronic inflammation at the
6 time you operate on a woman with ovarian cancer; is
7 that correct?
8   MS. O'DELL: Object to the form.
9   THE WITNESS: Yes, that's true.
10 BY MS. BOCKUS:
11   Q. Why would you no longer see the signs of
12 chronic inflammation at the time of her surgery for
13 ovarian cancer?
14   A. One, I'm not sure we know the signs that a
15 surgeon would identify as chronic inflammation to my
16 naked eye or to my field.
17   Two, most of the time in women with ovarian
18 cancer, three-quarters of the women I take care of
19 have cancer spread throughout their abdomen and
20 pelvis, with cancer everywhere, so that -- I mean, we
21 don't -- I don't know how to identify chronic
22 inflammation. I suggested that ascites has something
23 to do with inflammation but not always.
24   Q. And the ascites could come from the cancer
25 itself; correct?

Page 317

1   A. Yes.
2   Q. What would signs of chronic inflammation in
3 the fallopian tubes be?
4   MS. O'DELL: Object to the form.
5   THE WITNESS: I don't think there's any
6 signs that I'm aware of that recognize -- or would be
7 identified as chronic inflammation.
8 BY MS. BOCKUS:
9   Q. Is chronic inflammation something that could
10 be identified by a pathologist?
11   A. It might be.
12   Q. Do you know whether there have been any
13 studies looking at -- looking for signs of chronic
14 inflammation in women whose fallopian tubes have been
15 removed as part of any of the studies that you cite?
16   MS. O'DELL: Object to the form.
17   THE WITNESS: I'm sorry. They've had
18 their fallopian tubes removed?
19 BY MS. BOCKUS:
20   Q. And looked at by a pathologist, yes. And
21 it's reported in the studies.
22   A. Signs of chronic inflammation of the
23 fallopian tube? I'm not aware of that, no.
24   Q. Okay. Would you expect a woman who is using
25 talcum powder regularly to have signs of inflammation

Daniel L. Clarke-Pearson, M.D.

Page 318

1 in her fallopian tubes?
2   MS. O'DELL: Objection. Form.
3   THE WITNESS: Again, the signs of
4 chronic inflammation are vague and not well defined in
5 terms of what a pathologist would see. If they did
6 molecular testing -- for example, the reason we now
7 believe that most ovarian cancers arise in the
8 fallopian tube is by doing molecular testing of the
9 fallopian tube and seeing p53 mutations and early
10 cancers arising from the fallopian tube that then
11 metastasize to the ovary in the peritoneal cavity. So
12 that's a molecular biology approach that pathologists
13 don't usually do unless it's in a research setting.
14 BY MS. BOCKUS:
15   Q. Is it your belief that pathologists cannot
16 identify chronic inflammation in tissue samples that
17 they examine?
18   MS. O'DELL: Objection. Form.
19   THE WITNESS: I think they can identify
20 it on some occasions on H&E slides. Is that what
21 you're talking about?
22 BY MS. BOCKUS:
23   Q. Yes.
24   A. I think they can see it sometimes.
25   Q. And do you know if chronic inflammation is

Page 319

1 reported as existing in the fallopian tubes in any of
2 the studies that you have cited in your report?
3   MS. O'DELL: Objection. Asked and
4 answered.
5   THE WITNESS: Not that I'm aware of,
6 no.
7 BY MS. BOCKUS:
8   Q. I'm going to be jumping around a lot, and I'm
9 just going to apologize in advance for that --
10   A. Okay.
11   Q. -- but so much of what I was going to ask you
12 has already been covered.
13   Did I understand you correctly to say that
14 it is your belief that age causes ovarian cancer?
15   A. Age causes ovarian cancer?
16   Q. Yes.
17   A. Age allows time for mutations to occur; and,
18 therefore, ovarian cancer comes from that.
19   Q. Do you know what the relative risk of ovarian
20 cancer is for a woman in her 60s compared to a woman
21 in her 30s?
22   A. I'd have to look at some statistical tables.
23 I'm sure it's available.
24   Q. But it's greater than three or four; correct?
25   MS. O'DELL: Object to the form.

Page 320

1   THE WITNESS: I'm not sure how much
2 greater. It's greater as women age.
3 BY MS. BOCKUS:
4   Q. You indicated that not using birth control
5 pills causes ovarian cancer.
6   Did I understand you correctly?
7   MS. O'DELL: Object to the form.
8   THE WITNESS: It allows, more likely
9 than not, more mutations to occur as the patient
10 ovulates rather than having ovulation suppression by
11 birth control pills.
12 BY MS. BOCKUS:
13   Q. Okay. Do you believe that that mechanism is
14 supported in light of the fact that it is now believed
15 that cancers originate in the fallopian tubes?
16   A. Yes, I think it's hormonal changes in the
17 fallopian tubes as well as the ovary.
18   Q. Okay. Do you know to what -- what are the
19 odds ratios for a woman developing ovarian cancer who
20 has never used birth control pills compared to women
21 who have?
22   A. There's one statistic, I think, that is
23 pretty well agreed upon is that women who used birth
24 control pills for five years have about a 50 percent
25 reduction in the lifetime risk of ovarian cancer.

Page 321

1   Q. In your report on page 4, at the bottom, you
2 talk about EOC risk factors.
3   Can you see where I'm talking about?
4   A. Yes, ma'am.
5   Q. And you say (as read):
6   "The lifetime risk of developing
7   ovarian cancer is 39 to 46 percent
8   in BRCA1 carriers."
9   Did I read that correctly?
10   A. Yes.
11   Q. So does that come out to 390 to 460 women per
12 thousand who carry the BRCA1 gene mutation will
13 develop ovarian cancer in their lifetime?
14   MS. O'DELL: Objection to form.
15   THE WITNESS: Give me a second to do
16 the math. So if we had a thousand women, in their
17 lifetime, 390 would develop ovarian cancer.
18 BY MS. BOCKUS:
19   Q. Okay. Somewhere between 390 and 460?
20   A. Yes. I just did the math for one, but yes.
21   Q. Okay. And then going on, women who carry the
22 BRCA2 mutation, it would be 110 to 270 out of 1,000 in
23 their lifetime would develop ovarian cancer; is that
24 correct?
25   A. Yes.

Daniel L. Clarke-Pearson, M.D.

Page 322

1   MS. O'DELL: For women with BRCA2?
2   MS. BOCKUS: Yes. For women with
3 BRCA2. I thought I made that qualification.
4 BY MS. BOCKUS:
5   Q. And then you say (as read):
6   "This is compared to the
7   1.3 percent lifetime risk in
8   noncarriers."
9   Correct?
10   A. That's correct.
11   Q. So in other words, 13 women out of 1,000,
12 approximately, in the US will develop ovarian cancer
13 in their lifetime?
14   MS. O'DELL: Objection to form.
15 BY MS. BOCKUS:
16   Q. Is that what that means?
17   A. Yes.
18   MS. O'DELL: Objection to form.
19 BY MS. BOCKUS:
20   Q. And it's your opinion that -- and that's
21 all-comers; right? That's women who have had
22 children, women who haven't had children, et cetera?
23   A. Yes.
24   Q. That's the entire population?
25   A. But that don't have these BRCA mutations.

Page 323

1   Q. Correct. Fair enough.
2   So, as I understand it, it is your opinion
3 that the use of body powders, talcum body powders,
4 increases a woman's risk by about 30 percent. Is that
5 correct?
6   A. That's what the epidemiology says, yes.
7   Q. Okay. So does that mean that, instead of 13
8 out of 1,000 women who use talcum powder, then you
9 would expect to see 17 out of 1,000 who would develop
10 ovarian cancer in their lifetime?
11   MS. O'DELL: Object to the form.
12   THE WITNESS: I'd have to do the math,
13 but that sounds about right.
14 BY MS. BOCKUS:
15   Q. And out of those 17 per thousand, 13 would
16 have developed it anyway; correct?
17   MS. O'DELL: Object to the form.
18   THE WITNESS: Yes.
19 BY MS. BOCKUS:
20   Q. And do you know of any methodology that would
21 allow you to identify which of the 4 out of 17
22 developed ovarian cancer because of their use of talc
23 as opposed to just being on this planet and living a
24 certain number of years?
25   MS. O'DELL: Object to the form.

Page 324

1   THE WITNESS: Being on the planet is
2 the 1.3 percent, or the 13 out of 1,000.
3 BY MS. BOCKUS:
4   Q. Correct.
5   A. Being exposed to talc adds the other 4, if
6 your math is right --
7   Q. Okay. But do you know of any way that you or
8 anyone else can say, in this group of 17 women who
9 have ovarian cancer who used talcum powder, it's these
10 4 who developed it because of their talcum powder use
11 versus the 13 that we know would have been diagnosed
12 with ovarian cancer whether they ever used talc or
13 not?
14   MS. O'DELL: Objection. Incomplete
15 hypothetical.
16   THE WITNESS: So this is a hypothetical
17 that 1,000 women used talcum powder, and we knew, if
18 they hadn't used talcum powder, that 1 point -- that
19 13 of them would develop it, and then the other 4
20 develop it because, in my opinion, they used talcum
21 powder?
22 BY MS. BOCKUS:
23   Q. Right, because that's the difference between
24 the background rate and the rate that, it's your
25 opinion, is associated with talc use; correct?

Page 325

1   A. So do I know which one of those -- what
2 number are we up to now?
3   Q. The 4 out of 17.
4   A. -- the 4 out of 17 --
5   Q. Yes.
6   A. -- was caused by talcum powder?
7   Q. Right.
8   A. I don't think I can say that.
9   Q. Do you know of any methodology that would
10 allow someone to identify which of the 4 out of 17
11 were associated with their talc use versus associated
12 with just living that long?
13   MS. O'DELL: Objection to form.
14   THE WITNESS: I'm not aware of any --
15 if you're talking about biomarkers or something else,
16 I'm not aware of any that would distinguish between
17 cancer caused by talc and cancer caused by age alone.
18 BY MS. BOCKUS:
19   Q. Okay. And if one were to guess, they would
20 be mistaken two times out of three; correct?
21   MS. O'DELL: Object to the form.
22   THE WITNESS: To guess about what?
23 BY MS. BOCKUS:
24   Q. Which of the 17 had ovarian cancer because of
25 their talc use as opposed to because they would have

Daniel L. Clarke-Pearson, M.D.

Page 326

1 gotten it anyway?
2   MS. O'DELL: Object to the form.
3   THE WITNESS: I'm not quite sure
4 I understand where you're going or what the question
5 is. I think the answer is we don't -- we won't -- we
6 can't identify which one of those patients that have
7 ovarian cancer because they all -- your hypothetical
8 is that they all were exposed to talc.
9   MS. O'DELL: I don't think that was her
10 hypothetical.
11   THE WITNESS: Okay. Well, then I've
12 lost this.
13 BY MS. BOCKUS:
14   Q. As I under -- well, let me just move on.
15   When women go swimming in a swimming pool,
16 does chlorinated water go into their uterus?
17   A. Goes into their vagina.
18   Q. That wasn't my question. Does it go to their
19 uterus?
20   A. Probably not.
21   Q. Why not?
22   A. I don't know the answer to that question.
23   Q. When women go swimming in the ocean, does
24 saltwater go into their uterus?
25   A. Not usually, no.

Page 327

1   Q. Why not?
2   A. It just doesn't.
3   Q. Is there something blocking the uterus from
4 the vagina?
5   A. The cervix is there, and there is mucus in
6 the cervix at certain times. I think the other, to
7 follow up on your question with a little bit better
8 answer, is that exposure to the water is limited.
9 It's not like the patient's in the water for hours,
10 day after day after day.
11   Q. That really wasn't my question.
12   A. Okay.
13   Q. My question has to do with the passage of any
14 kind of particles from outside the human body to
15 inside the human body -- the female body.
16   A. Okay.
17   Q. Is it your opinion that particles contained
18 in bathwater make their way into the fallopian tubes?
19   A. I don't have an answer -- answer or opinion
20 on that.
21   Q. Same question for swimming pool water.
22   A. Likewise.
23   MS. O'DELL: Objection to form.
24 BY MS. BOCKUS:
25   Q. Do you know whether there's an increased

Page 328

1 incidence of ovarian cancer in women who have been
2 competitive swimmers?
3   A. Not that I'm aware of.
4   Q. Those women clearly will have spent hours a
5 day, every day, in a swimming pool for many years of
6 their life; correct?
7   A. Yes.
8   Q. And you would expect, would you not, if
9 particles from outside a woman's body could freely
10 move into the inside of her body, that the chlorine
11 and other particles found in a swimming pool would
12 make their way to their ovaries; correct?
13   A. They could. But if they're not carcinogens,
14 then they wouldn't cause any problem.
15   Q. Would any foreign body that makes its way to
16 its ovary -- to a woman's ovary cause a foreign body
17 reaction?
18   A. Not necessarily.
19   Q. What foreign particle could make its way to a
20 woman's ovary and not cause a foreign body reaction?
21   MS. O'DELL: Objection to the form.
22   THE WITNESS: I think that those that
23 don't cause inflammation, those that are not cleared.
24 We talked about cornstarch earlier in today's
25 proceedings, and cornstarch seems not to cause an

Page 329

1 inflammatory reaction. It gets cleared by the immune
2 system, and it dissolves.
3 BY MS. BOCKUS:
4   Q. Does cornstarch make it to the ovary?
5   A. Cornstarch has been documented to get to the
6 ovary, yes.
7   Q. Has it been associated with foreign body
8 reaction in the ovary?
9   A. Not that I'm aware of.
10   Q. Do you know whether pelvic mesh causes
11 ovarian cancer?
12   A. Mesh?
13   Q. Yes.
14   A. Not that I'm aware of.
15   Q. Is pelvic mesh a foreign body?
16   A. Yes. It's in the vagina or -- yeah, it's
17 placed in the vagina, not in the peritoneal cavity per
18 se.
19   Q. Does pelvic mesh cause chronic inflammation?
20   A. Not that I'm aware of. I think it causes
21 acute inflammation and an ingrowth of fibroblasts and
22 fibrous tissue to cause -- to get the result that the
23 surgeon wants and the patient wants.
24   Q. Just because something is classified as a
25 carcinogen doesn't mean it's carcinogenic to every

Daniel L. Clarke-Pearson, M.D.

Page 330

1 organ in the body; correct?
2   A. I think that's fair to say.
3   Q. And I think you told us previously that, to
4 your knowledge, you're not aware of nickel, chromium,
5 or cobalt ever being identified as carcinogenic to the
6 ovary; correct?
7   A. I'm not aware that anybody's ever tested that
8 hypothesis.
9   Q. Did you look at the IARC classifications of
10 those three heavy metals?
11   A. Yes.
12   Q. And did you see where IARC did not identify
13 that they were carcinogenic to the ovary?
14   MS. O'DELL: Objection to form.
15   THE WITNESS: Right. I'm not sure that
16 there's any data, going back to my answer to my last
17 question, where that's ever been tested. So two of
18 those heavy metals are considered carcinogens, but not
19 specifically to the ovary because they haven't been
20 tested in the ovary.
21 BY MS. BOCKUS:
22   Q. So without that -- without those tests, you
23 can't say one way or the other whether those heavy
24 metals, the three you identify in your report,
25 increase the risk of ovarian cancer, can you?

Page 331

1   MS. O'DELL: Object to the form.
2   THE WITNESS: I think they're contained
3 within Johnson's baby powder.
4 BY MS. BOCKUS:
5   Q. That wasn't my question.
6   Without science to support that, you cannot
7 say that these three heavy metals that you identify in
8 your report cause or contribute to cause ovarian
9 cancer; correct?
10   MS. O'DELL: Object to the form.
11   THE WITNESS: I think they're in
12 Johnson baby powder and the baby powder causes ovarian
13 cancer. So something amongst that, including the
14 heavy metals, is contributing to the onset of ovarian
15 cancer.
16 BY MS. BOCKUS:
17   Q. And you're comfortable saying that without
18 any science to support it; correct?
19   MS. O'DELL: Objection to form.
20   THE WITNESS: The science is the
21 epidemiology of increased risk of ovarian cancer in
22 women that are exposed to Johnson baby powder.
23 BY MS. BOCKUS:
24   Q. Did I understand your testimony previously
25 that it is your opinion that the Women's Health

Page 332

1 Initiative is a poorly designed, poorly executed
2 study?
3   MS. O'DELL: Object to the form.
4   THE WITNESS: Yes.
5 BY MS. BOCKUS:
6   Q. Is it your opinion that the Nurses' Health
7 Study is a poorly designed, poorly executed study?
8   MS. O'DELL: Object to the form.
9   THE WITNESS: With regard to the
10 detection of ovarian cancer being caused by perineal
11 use of talcum powder, yes.
12 BY MS. BOCKUS:
13   Q. Is it your opinion that the Gonzalez Sister
14 Study is a poorly designed, poorly executed study?
15   A. Yeah. That's the worst one.
16   Q. You have testified -- and this certainly
17 would be part of your practice to understand -- that
18 we now know that HPV causes cervical cancer; correct?
19   A. That's correct.
20   Q. What is the odds ratio of developing cervical
21 cancer in women who have HPV -- or who have had HPV
22 versus those who have not?
23   A. HPV is nearly 100 percent -- let me turn this
24 back around.
25   Women with squamous cell carcinoma of the

Page 333

1 cervix, which is the most common type, almost all --
2 as close to 100 percent as possible -- have been
3 infected with HPV.
4   Q. And that allows the scientific and medical
5 community to conclude with consensus that HPV causes
6 cervical cancer; correct?
7   A. Yes, but not in all women that are infected
8 with HPV.
9   Q. There is no similar factor for ovarian cancer
10 as closely linked as HPV is to cervical cancer, is
11 there?
12   MS. O'DELL: Objection to form.
13   THE WITNESS: I'm not sure I understand
14 the question.
15 BY MS. BOCKUS:
16   Q. Because it wasn't a very good one.
17   A. Okay.
18   Q. You indicated that close to 100 percent of
19 all women who develop a specific -- the most common
20 type of cervical cancer have had HPV; correct?
21   A. That's correct.
22   Q. There is nothing even close to that in terms
23 of an exposure and ovarian cancer; correct?
24   A. Yes, I would agree.
25   Q. Do you know what percentage of sperm make it

Daniel L. Clarke-Pearson, M.D.

Page 334

1 to the fallopian tube from a single ejaculation?
2   A. I don't.
3   Q. You know that that's been studied; correct?
4   A. I don't know that. The last time I did any
5 reproductive endocrinology was in 1975. So I don't
6 know what's --
7   Q. Let me ask you --
8   A. -- been studied.
9   Q. I apologize. I didn't mean to interrupt.
10   A. Yes.
11   Q. Do you have any reason to believe that a talc
12 particle would fare better than a sperm in terms of
13 its chances of making it from the vagina to the ovary?
14   MS. O'DELL: Object to the form.
15   THE WITNESS: No.
16 BY MS. BOCKUS:
17   Q. Do you think that it's probably that fewer
18 talc particles -- or a smaller percentage of talc
19 particles deposited into the vagina would make it to
20 the ovary than percentage of sperm?
21   A. I don't have an opinion.
22   Q. Okay. With regard to studies by Dr. Saed, do
23 you believe that it would have been appropriate for
24 Dr. Saed to indicate on those studies that his
25 research was being funded by plaintiffs' lawyers in

Page 335

1 this litigation?
2   MS. O'DELL: Object to the form.
3   THE WITNESS: I'm not sure I understand
4 exactly what was his funding.
5 BY MS. BOCKUS:
6   Q. For the studies that you're relying on, the
7 Saed studies that you have relied on in your report.
8   A. I'm not aware of the extent of the funding,
9 if it was from the attorneys -- the plaintiffs'
10 attorneys.
11   Q. Assuming that the evidence will show that the
12 funding for Dr. Saed's experiments came from
13 plaintiffs' attorneys, would it be appropriate and
14 ethical for a physician to reveal that that's the
15 source of their funding?
16   MS. O'DELL: Objection to form.
17   THE WITNESS: So peer-reviewed journals
18 have certain conflict of interest statements and
19 disclosures that are asked as part of the peer review
20 process of accepting a manuscript. So I'm not sure
21 what the policies are of this particular journal.
22 BY MS. BOCKUS:
23   Q. So does such a conflict of interest only have
24 to be revealed if it's the policy of the journal?
25   MS. O'DELL: Objection to form.

Page 336

1   THE WITNESS: I think the journal, if
2 it's going to publish, would want to make sure that
3 they are publishing information that's correct and,
4 you know, through the peer review process, and also
5 any conflicts of interest are declared, any sources of
6 funding are usually declared, including grants from
7 National Institutes of Health, for example.
8 BY MS. BOCKUS:
9   Q. When Dr. Saed placed talc on these cultured
10 ovarian cancer cells, one of the findings that he
11 reported was that it increased the level of CA-125;
12 correct?
13   A. Yes.
14   Q. You would agree that CA-125 is raised by many
15 things; correct?
16   A. Yes, including inflammation -- in particular
17 inflammation in terms of a false positive CA-125.
18   Q. It can be raised by pregnancy; is that right?
19   A. Yes.
20   Q. Can be raised by cirrhosis of the liver?
21   A. Yes.
22   Q. Can be raised by uterine fibroids; correct?
23   A. Yeah --
24   Q. By all kinds of things?
25   A. -- among other things, yes.

Page 337

1   Q. And Dr. Saed did not use any positive or
2 negative controls in his study, did he?
3   MS. O'DELL: Objection. Form.
4   THE WITNESS: He did use controls in
5 his study.
6 BY MS. BOCKUS:
7   Q. Did Dr. Saed use any controls in which he
8 applied a -- something like glass beads to the same
9 tissue to see what the reaction would be compared to
10 the talc he was applying?
11   MS. O'DELL: Objection to form.
12   THE WITNESS: So applying glass -- I'm
13 not a laboratory scientist, but putting glass beads
14 into a culture plate, for example? So that would be
15 potentially another inflammatory product, so I don't
16 know why one would put glass beads into the control
17 plate.
18   He has controls in all of his tables here
19 (indicating). It's just the medium that the talc is
20 suspended in. So the medium didn't cause the changes
21 that he demonstrates in these cancer cells and these
22 epithelial cells. It was the talc that caused the
23 changes. That's why you do a control.
24 BY MS. BOCKUS:
25   Q. But a -- but to do a control with regard

Daniel L. Clarke-Pearson, M.D.

Page 338

1 to -- to determine whether talc causes these cells to
2 react differently than other items that have
3 previously been shown not to cause inflammation in the
4 cells, you would need to add something in addition to
5 the medium; correct?
6   MS. O'DELL: Objection to form.
7   THE WITNESS: No. That's what a
8 control is. Why would you add anything? That would
9 be a third experiment. You've got your controls and
10 now your glass beads and now your talc.
11 BY MS. BOCKUS:
12   Q. Is it your understanding that glass beads
13 would cause inflammation to the ovarian epithelial?
14   A. I don't know what they do. I don't know why
15 one would put glass beads in a control.
16   Q. Other than the medium, did Dr. Saed
17 include -- did he do any test to determine whether
18 other particulate would cause the exact same reaction
19 as the talc?
20   A. I don't think that was part of his
21 experimental design.
22   Q. Do you think that would have been an
23 appropriate experimental design to determine if talc
24 elicited a response different than any other foreign
25 particulate?

Page 339

1   MS. O'DELL: Object to the form.
2   THE WITNESS: Oh, you could do an
3 extensive experiment of all kinds of particulates and
4 compare it with talc. That wasn't the question he was
5 trying to ask. I'm not quite sure where you're going
6 with this. I mean...
7 BY MS. BOCKUS:
8   Q. To determine whether the changes that he
9 noted actually cause cancer would take more steps;
10 correct?
11   A. Yes. He's showing --
12   MS. O'DELL: Object to the form.
13   THE WITNESS: -- that there's gene
14 mutations. They are the first step -- or the next
15 step towards cancer.
16 BY MS. BOCKUS:
17   Q. And all of our -- we all have gene mutations
18 going on in our bodies every day; correct?
19   A. Yes. A little scary.
20   Q. And we all have -- thank God, the way we're
21 put together, there are systems in place that detect
22 gene mutations and kill them; correct?
23   A. Apoptosis. Yes.
24   Q. And so the fact that a gene mutation is
25 caused in a Petri dish is a long ways from proving

Page 340

1 that that particulate -- in this case, talc -- causes
2 cancer; correct?
3   MS. O'DELL: Object to the form.
4   THE WITNESS: It doesn't -- it's not
5 conclusive, but it certainly is a step in the process
6 leading towards cancer.
7 BY MS. BOCKUS:
8   Q. And there are specific tests that can be done
9 for genotoxicity; correct?
10   Are you familiar with those --
11   A. I'm not familiar with what that exactly
12 means.
13   Q. Have you seen studies where, in the lab, they
14 have started this process, such as Dr. Saed did with
15 causing a single gene mutation, and then implanting
16 that tissue into a lab animal to see if it actually
17 grows into a cancer?
18   MS. O'DELL: Object to the form.
19   THE WITNESS: I'm not aware of that,
20 but it's certainly -- I presume it's possible to do
21 something like that, but I'm not sure.
22 BY MS. BOCKUS:
23   Q. I think you've answered this question. And
24 if you have, I apologize.
25   What is the threshold response for talc?

Page 341

1   MS O'DELL: Object to the form
2   THE WITNESS: The threshold response
3 that would induce cancer, I presume is what you're
4 really asking?
5 BY MS BOCKUS:
6   Q Yes, sir Thank you
7   A I don't think we know that
8   MS BOCKUS: That's all that I have
9 Thank you
10   THE WITNESS: Thank you
11   MS BOCKUS: I'll cede back my last 15
12 minutes to the other defense counsel who are here
13   MS O'DELL: Do you have questions?
14   MR BILLINGS-KANG: I don't think so,
15 no
16   MS O'DELL: Do you have questions?
17   MR ZELLERS: No further questions
18   MR MIZGALA: I want to ask a question
19   MR ZELLERS: Please do
20   CROSS-EXAMINATION BY COUNSEL FOR THE DEFENDANT PTI
21 BY MR MIZGALA:
22   Q Doctor, on page 2 of your report, at the
23 bottom --
24   A Yes
25   Q -- you write (as read):

Daniel L. Clarke-Pearson, M.D.

Page 342

1   "I approached each article
2   objectively and critically,
3   assessing for factors such as
4   design, power, reputation of the
5   authors, quality of the journal,
6   and potential biases."
7   Correct?
8   A. Yes, that's what I wrote.
9   Q. Where is that -- where is that written down?
10 Where is it compiled?
11   A. Where is what compiled?
12   Q. All those things that you assessed? Did you
13 reduce that to writing anywhere?
14   A. No. I mean, these are the articles
15 I identified and reviewed and assessed (indicating).
16   Q. Okay. So you don't have a spreadsheet or
17 something of all these factors that you assessed?
18   A. No.
19   MS. O'DELL: Objection to form.
20   THE WITNESS: No.
21 BY MR. MIZGALA:
22   Q. In your head?
23   A. In my head at the time, and I chose articles
24 that I thought were appropriate to put into my report.
25   MR. MIZGALA: Okay. No further

Page 343

1 questions.
2   MS. O'DELL: Let's go off the record.
3   THE VIDEOGRAPHER: Going off record at
4 5:23 p.m.
5   (Recess taken from 5:23 p.m. to 5:40 p.m.)
6   THE VIDEOGRAPHER: Back on the record
7 at 5:40 p.m.
8   CROSS-EXAMINATION BY COUNSEL FOR THE PLAINTIFFS
9 BY MS. O'DELL:
10   Q. Dr. Clarke-Pearson, I have just a few
11 questions to ask you.
12   First, let me ask you, in regard to
13 asbestos, can asbestos be inhaled and cause ovarian
14 cancer?
15   MR. ZELLERS: Objection to form.
16   THE WITNESS: Yes.
17   Yes. IARC has deemed that true, to be the
18 case that it can cause ovarian cancer by inhalation.
19 BY MS. O'DELL:
20   Q. And, similarly, can fibrous talc be inhaled
21 and cause ovarian cancer?
22   MR. ZELLERS: Objection. Form.
23   THE WITNESS: Yes. The same answer.
24 BY MS. O'DELL:
25   Q. You were asked a series of questions about --

Page 344

1 and they were hypotheticals, as I recall -- regarding
2 specific patients and the cause or causes of their
3 ovarian cancer.
4   In regard to a woman who has potentially,
5 say, a BRCA mutation -- maybe she's of a certain
6 age -- and she's a routine user of talcum powder such
7 as Johnson's baby powder, do you have an opinion as to
8 what the causes of her cancer would be?
9   MR. ZELLERS: Objection. Form.
10   THE WITNESS: So several causes, but
11 the talcum powder would have to be considered a
12 contributing cause to her ovarian cancer.
13 BY MS. O'DELL:
14   Q. For a woman who has -- in whom there's not
15 been identified a known risk factor but she is a
16 routine user of talcum powder such as baby powder or
17 Shower to Shower, do you have an opinion as to what
18 one of the causes of her cancer -- ovarian cancer
19 would be?
20   MR. ZELLERS: Objection. Form.
21   THE WITNESS: What I've been trying to
22 say all day is the Johnson & Johnson baby powder
23 causes ovarian cancer. In this particular patient, it
24 is a significant contributing cause.
25   MS. O'DELL: I have nothing further,

Page 345

1 Doctor. Thank you.
2   THE WITNESS: Okay. Thank you.
3   FURTHER EXAMINATION BY COUNSEL FOR THE
4   JOHNSON & JOHNSON DEFENDANTS
5 BY MR. ZELLERS:
6   Q. The asbestos studies that you referred to
7 earlier dealing with inhalation, those were
8 occupational studies; correct?
9   MS. O'DELL: Object to the form.
10   THE WITNESS: Yes.
11   MR. ZELLERS: Okay. I have no further
12 questions.
13   MS. BOCKUS: I have one.
14   FURTHER EXAMINATION BY COUNSEL FOR THE
15   DEFENDANT IMERYS
16 BY MS. BOCKUS:
17   Q. Doctor, are you aware of any study that
18 indicates that women who carry a BRCA gene mutation
19 and uses -- and has a lifetime history of using talcum
20 powder is at a higher risk of developing ovarian
21 cancer than women who have the BRCA gene mutation and
22 have never used talcum powder?
23   MS. O'DELL: Objection to form.
24   THE WITNESS: It would be my opinion
25 that talcum powder would increase the patient's chance

Daniel L. Clarke-Pearson, M.D.

Page 346

1 of having ovarian cancer. I'm not aware of any study
2 that's been able to investigate that to date.
3 BY MS. BOCKUS:
4   Q. That is something that could be investigated;
5 correct?
6   MS. O'DELL: Object to the form.
7   THE WITNESS: In a case-control study,
8 yes.
9 BY MS. BOCKUS:
10   Q. But to your knowledge, it's never been
11 reported; correct?
12   A. Not that I'm aware of.
13   MS. BOCKUS: That's all I have.
14   THE WITNESS: Thank you, everybody.
15   MR. ZELLERS: Thank you, Doctor.
16   THE VIDEOGRAPHER: Just one second.
17   This concludes the deposition of Dr. Daniel
18 Clarke-Pearson. Time going off the record is
19 5:44 p m.
20   (Whereupon, at 5:44 p m., the deposition ceased.
21   Signature was reserved.)
22
23
24
25

Page 348

1   E R R A T A
2 CASE NAME: TALCUM POWDER LITIGATION MDL NO 2738CASE
3 WITNESS NAME: DANIEL L CLARKE-PEARSON, M D
4 CASE NUMBER: 16-2738 (FLW)(LHG)
5 PAGE LINE   READS   SHOULD READ
6 ___ ___ _____ _____
7 ___ ___ _____ _____
8 ___ ___ _____ _____
9 ___ ___ _____ _____
10 ___ ___ _____ _____
11 ___ ___ _____ _____
12 ___ ___ _____ _____
13 ___ ___ _____ _____
14 ___ ___ _____ _____
15 ___ ___ _____ _____
16 ___ ___ _____ _____
17 ___ ___ _____ _____
18 ___ ___ _____ _____
19 ___ ___ _____ _____
20 ___ ___ _____ _____
21 ___ ___ _____ _____
22 ___ ___ _____ _____
23 ___ ___ _____ _____
24 ___ ___ _____ _____
25 ___ ___ _____ _____

Page 347

1   ACKNOWLEDGMENT OF DEPONENT
2   I, DANIEL L. CLARKE-PEARSON, M.D., do hereby
3 acknowledge that I have read and examined the foregoing
4 testimony, and the same is a true, correct, and complete
5 transcription of the testimony given by me, and any
6 corrections appear on the attached errata sheet signed
7 by me.
8
9 _____ _____
10   (DATE)   (SIGNATURE)
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25

Page 349

1 STATE OF NORTH CAROLINA)
2   ) C E R T I F I C A T E
  COUNTY OF ORANGE   )
3   I, Sophie Brock, Court Reporter and Notary
4 Public, the officer before whom the foregoing proceeding
5 was conducted, do hereby certify that the witness(es)
6 whose testimony appears in the foregoing proceeding were
7 duly sworn by me; that the testimony of said witness(es)
8 were taken by me to the best of my ability and
9 thereafter transcribed under my supervision; and that
10 the foregoing pages, inclusive, constitute a true and
11 accurate transcription of the testimony of the
12 witness(es).
13   I do further certify that I am neither counsel
14 for, related to, nor employed by any of the parties to
15 this action, and further, that I am not a relative or
16 employee of any attorney or counsel employed by the
17 parties thereof, nor financially or otherwise interested
18 in the outcome of said action.
19   This, the 6th day of February, 2019.
20
21
22
                    _____
23
              Sophie Brock, RPR, RMR, RDR, CRR
24   Notary Number: 200834000001
25

Exhibit 73

UNITED STATES DISTRICT COURT
DISTRICT OF NEW JERSEY
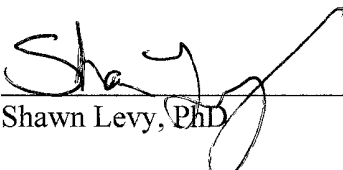
IN RE JOHNSON & JOHNSON TALCUM POWDER PRODUCTS MARKETING, SALES PRACTICES, AND PRODUCTS LIABILITY LITIGATION	MDL NO. 16-2738 (FLW) (LHG)
THIS DOCUMENT RELATES TO ALL CASES	

RULE 26 EXPERT REPORT OF
SHAWN LEVY, PHD

Date: November 16, 2018

Shawn Levy, PhD

I.   Qualifications and Background

I am a founding director of and a faculty investigator with the Genomic Services Laboratory at the HudsonAlpha Institute for Biotechnology. My focus is on use of high performance genotyping and sequencing technologies as support for plant and animal phylogenetic studies and translational and clinical-based projects. A portion of my research entails using whole-genome sequencing to identify genetic markers associated with specific health conditions.

I serve as executive director of the HudsonAlpha Clinical Services Laboratory, LLC, which I launched in 2014. I am adjunct faculty in the department of genetics and department of epidemiology at the University of Alabama at Birmingham, adjunct faculty in the department of biological Sciences at the University of Alabama at Huntsville, and serve as an ad hoc reviewer for scientific journals including Nature, Nature Genetics, Science, Cell, Genome Research and several others. I have been a co-chair of the Genomics Working Group of the American Medical Informatics Association, a community of scientists and health care professionals that work to facilitate collaboration and share knowledge across a continuum, from basic and applied research to the consumer and public health arenas.

Prior to joining HudsonAlpha in 2009, I was a faculty member at Vanderbilt University Medical Center with appointments in the Department of Molecular Physiology and Biophysics and the Department of Biomedical Informatics. I was the founding director of the Vanderbilt Microarray Shared Resource where I served as Director for 9 years. I received my PhD in biochemistry and completed a postdoctoral fellowship in genetics at Emory University in Atlanta, where I set up a microarray facility at the Emory Center for Molecular Medicine. My education, training, and experience are further set forth in my Curriculum Vitae (CV), which is attached to this report as **Exhibit A**.

As detailed in my CV, my research activities have examined a number of basic questions in human cancer such as the role of viral infection in head and neck cancer, the role of genetic mutation in risk for secondary cancer events following initial treatment, the genetics of B-cell

lymphoma, hepatosplenic T-cell lymphoma and malignant melanoma, and the role of STAT3 in triple-negative breast cancer. As the founding and Executive Director of the HudsonAlpha Clinical Services Laboratory, I also have interests and responsibilities in the clinical use of genetic testing for cancer risk and treatment stratification. HudsonAlpha launched the Information is Power campaign and has provided genetic testing for breast and ovarian cancer risk to women across the state of Alabama free of charge. My lab has also supported the Alabama Genomics Health Initiative that tests for genetic risks and carrier status for a number of diseases, including breast and ovarian cancer. This body of work in basic and clinical research in combination with earlier epidemiological work in the Shanghai Women's Health study provides the experience, education and expertise to develop this report.

I have been retained to describe the role of genetics in the pathogenesis of cancer in general and specifically ovarian cancer. Further, I have been asked to assess whether perineal use of talcum powder products induces a biologically plausible mechanism or mechanisms that result in ovarian cancer.

My report consists of a review and my conclusions regarding this cause-and-effect relationship. My opinions are based on my assessing and weighing the totality of the evidence, including relevant literature and available documentation, and my experience as a geneticist and scientific researcher.   Report references are listed at the end of this report, and a more comprehensive list of the documents and materials reviewed prior to formulating the opinion in this report is attached as **Exhibit B**. The methodology that I have used to reach my opinions in this case is generally accepted in the scientific community and is the same methodology that I use in my research and other professional activities.   All of my opinions stated below are held to a reasonable degree of scientific certainty. My opinions reflect my sole and independent judgment at the time of this report.

My billing rate is $500 per hour. I have not testified by deposition or at trial during the last four years.

2

II.   Cancer Overview

Cancer has become a descriptor that is ubiquitously used but describes an extremely complex and diverse collection of medical conditions. Cancer is also a word that represents an amazingly complicated and often misunderstood collection of diseases. At the most basic level, cancer can be described as a disease of unregulated cell growth but its simplicities end with that simple description. From the moment of conception until death, humans experience an unending cycle of cell growth, differentiation and death. As infants grow to children and then to adults, there are an array of growth processes that occur that represent the milestones of development and maturation. These processes are an orchestra of highly coordinated and regulated events with important checks and balances. When those highly regulated processes are defective or the checks and balances malfunction, the growth of the cells can become unregulated. Which tissue or cells become unregulated and exactly what process is defective defines the type of cancer and its progression. Cancer can be aggressive and highly metastatic when unregulated cells invade other parts of the body and destroy organs and tissues. Other types of cancer remain restricted to specific organs or cell types and may be less aggressive.

It is the DNA within our cells which provides the genetic code or instructions to create the cells, tissues, and organs that make a human. Subtle changes in that code lead to the diversity of people around the world, while more substantial changes in that code create the diversity of life forms around us, from the smallest bacteria to the largest plants and animals. All cells have one set of instructions that provides the information for cells to divide, tissues to grow and how cells should die.

III.   The Role of Gene Mutations in the Development of Cancer

At its fundamental level, cancer is caused by changes (mutations) to the DNA within cells. The DNA that makes up our genetic code is organized into a large number of individual genes, each of which contains a specific subset of instructions telling the cell what functions to perform, as well as how to grow and divide. Errors in the instructions can cause the cell to stop its normal function and may allow a cell to become cancerous. Mutations that cause cancer most commonly

3

disrupt the regulation of the cell cycle (i.e., stages of cell growth and division). The following classifications of mutations are those most commonly found in cancer, but many other gene mutations can contribute to causing cancer as well.

> Increasing cell growth and division. A gene mutation can initiate more rapid cell growth and division, resulting in many new cells that all have that same mutation. Proto-oncogenes are a group of genes that regulate cell growth, differentiation, division and death. When a proto-oncogene is mutated, it can become an oncogene that then instructs the cell to grow rapidly in an unregulated manner.

> Loss of growth inhibition. A gene mutation can result in the renewed growth of a cell that had previously stopped growing. Normal cells regulate their division so that the human body contains the appropriate number of each type of cell. When the tumor suppressor genes that provide this inhibitory control become mutated, cells become cancer cells and continue to grow and amass. An example of one such gene is *p53*, which is discussed in more detail below.

> Loss of DNA repair. Gene mutations can also affect the genes that proofread DNA and fix mutations before they can have a detrimental effect. DNA repair genes look for errors in a cell's DNA and make corrections. A mutation in a DNA repair gene may mean that other errors aren't corrected, leading cells to become cancerous through unchecked replication of damaged cells. Examples of DNA repair genes include *BRCA1* and *BRCA2* which are discussed in more detail below.

Another way of classifying gene mutations is by when they occur.

1) Inherited gene mutations: Inherited gene mutations are those mutations an individual is born with and that are present in all cells of the body. These types of mutations define traits and characteristics that have a family history. This type of mutation directly accounts for a small percentage of cancers. The indirect effects of this type of mutation is an area of active research. There are a growing number of genes and mutations that are known to increase the risk of cancer. *BRCA1* and *BRCA2* mutations and the increased risk for breast and ovarian cancer are two examples. While additional genes are being identified, the

4

percentage of individuals affected by mutations in those genes will be significantly less than those affected by *BRCA1* and *BRCA2*.

2) <u>Acquired (somatic) gene mutations</u>: Somatic mutations are acquired after birth. Most gene mutations that directly cause cancer occur after birth and aren't inherited. Gene mutations can be caused by a number of events or exposures. These include environmental exposures such as smoking, radiation, and cancer-causing chemicals (carcinogens). Biological and lifestyle exposures such as viruses, obesity, hormones, and chronic inflammation are also known to result in cancer-causing mutations. Each exposure type has its own mechanism in increasing risk for cancer. These mechanisms may be direct, such as radiation directly damaging DNA, as well as indirect, such as an external agent causing a cellular reaction or inflammatory response that then leads to DNA damage or mutation.

Both inherited and acquired gene mutations work together to cause cancer. While genetic testing has become commonplace for both assessing risk for cancer as well as directing treatment, the catalog of oncogenes, tumor suppressor genes, and DNA repair genes make genetic testing valuable and impactful for informing patients of their genetic risk for cancer. Genetic testing generally detects inherited mutations. Currently, genetic screening does not detect acquired gene mutations because they occur only in certain cells. Even if one has inherited a genetic mutation that predisposes one to cancer, that doesn't mean he or she is certain to get cancer. Rather, one or more additional gene mutations may be needed to cause cancer. The inherited gene mutation could instead make one more likely to develop cancer when exposed to a certain cancer-causing substance. Conversely, an individual may still develop cancer if they do not have mutations known to predispose one to cancer. Additionally, chemical and other environmental agents such as talcum powder products can interact with inherited mutations to cause ovarian cancer.

IV.   The Role of Genetics in Ovarian Cancer

Ovarian cancer is the major cause of death from gynecologic disease and the second most common gynecologic malignancy worldwide (Nunes and Serpa, 2018; Siegel, 2015; Torre, 2015). The term "ovarian cancer" is often used to include fallopian tubal, ovarian epithelial and peritoneal

cancers since the pathogenesis, treatment and clinical courses are similar. Researchers now believe that most of these cancers originate in the distal portion of the fallopian tube (Levanon, 2008). The significant mortality is primarily associated with late diagnosis and resistance to therapy (Bowtell, 2010). Epithelial ovarian cancer (EOC) includes most malignant ovarian neoplasms (Chan, 2006) that can be classified based on morphologic and molecular genetic features into the following types: serous (OSC; low and high grade), endometrioid (EC), clear cell (OCCC) and mucinous (MC) carcinomas.

Certain specific genetic and transcriptional signatures are associated with each histological subtype. Low-grade OSC cases generally have genetic alterations in BRAF, KRAS, NRAS, and Erb-B2 Receptor Tyrosine Kinase 2 (ERBB2); high-grade OSC has mutations in Tumor Protein P53 (TP53), BRCA1/2, Neurofibromin 1 (NF1), RB Transcriptional Corepressor 1 (RB1), and Cyclin Dependent Kinase 12 (CDK12) (Chan, 2006). Homologous recombination repair of DNA damage is defective in approximately 50% of high-grade serous cancers along with alterations in signaling pathways such as PI3/Ras/Notch/ FoxM1 (Nunes and Serpa, 2018).

Endometroid carcinoma (EC) subtypes involve mutations in AT-Rich Interaction Domain 1A (ARID1A), Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha (PI3KCA), Phosphatase And Tensin Homolog (PTEN), Protein Phosphatase 2 Scaffold Subunit Alpha (PPP2R1α), and mismatch repair deficiency. Ovarian clear cell carcinoma (OCCC) subtypes have been found with de novo expression of HNF1β (Mabuchi, 2009; Shen, 2013) as well as ARID1A, PI3KCA, PTEN, Catenin Beta 1 (CTNNB1) and PPP2R1α mutations. MC comprises tumors with mutations in KRAS and a high frequency of ERBB2 amplification with overexpression of mucin-coding genes (Banerjee and Kaye, 2013; Jayson, 2014).

In addition to inherited mutations, exposure to the environment can result in DNA changes, or acquired gene mutations, that lead to cancer. These sources can be from exposure to minerals such as asbestos or arsenic, chemical exposures such as benzene or formaldehyde and from natural radiation sources like radon or ultraviolet light. These exposures constantly damage human DNA. Fortunately, cells have robust DNA repair mechanisms to ensure DNA damage is repaired before the DNA is replicated. These "proofreading" mechanisms react to DNA damage and stop DNA

6

replication. The mechanisms involve checkpoint control proteins such as the p53 protein, which acts to stop the cell cycle if DNA is damaged, and thus to suppress production of tumors. Cells that do not express functional p53 protein exhibit high rates of mutation in response to DNA damage, accelerating the formation of tumors.

BRCA1 and BRCA2 proteins also function in the DNA repair pathway. *BRCA1* and *BRCA2* are normally expressed in the cells of breast and other tissue, where they help repair damaged DNA, or destroy cells if DNA cannot be repaired. They are involved in the repair of chromosomal damage resulting from double-strand breaks. *BRCA1* combines with other tumor suppressors, DNA damage sensors and signal transducers to form a large multi-subunit protein complex known as the BRCA1-associated genome surveillance complex (BASC). BRCA2 interacts with the RAD51 protein, also forming a complex that is vital for DNA repair.

Individuals can inherit mutations in *BRCA1*, *BRCA2* or *p53*,[1] and are termed "positive" for the gene mutation. Such mutations will detrimentally affect the ability to repair DNA or sense the presence of damaged DNA. These defects allow additional mutations to accumulate in cells and lead to a higher probability of cells becoming cancerous. *BRCA1*, *BRCA2* and *p53* mutations can also be acquired in certain cells. If those cells form a tumor, the cancerous tissue can be tested for these gene mutations.

BRCA mutations are inherited in an autosomal dominant fashion, meaning inheriting only one copy results in increased cancer risk. Some individuals with a mutation in the *BRCA1* or *BRCA2* gene will develop cancer during their lifetime, but others will not. Penetrance refers to the proportion of individuals with a genetic mutation who exhibit symptoms of the disorder. Where some carriers do not develop a disorder, as in the case of *BRCA* carriers, the condition is said to have incomplete penetrance. In such instances, additional genetic, environmental and lifestyle factors must be present for the disorder to manifest. The lifetime risk for ovarian cancer is approximately 40 percent for *BRCA1* carriers and 15 to 20 percent for *BRCA2* carriers (Berek et

---

[1] Genes consist of genetic information that code for functional proteins. Both the gene and the protein they code share the same alphanumeric name. To avoid confusion, genes are italicized in text and proteins are not. For example: *BRCA1* (gene) and BRCA1 (protein).

al., 2012; Paluch-Shimon et al., 2016). Therefore, the presence of mutations in the *BRCA* genes do not guarantee that carriers will get cancer. The presence of these mutations increases a person's risk of developing cancer when exposed to a carcinogen (Park, 2018; Vitonis, 2011; Wu, 2015).

Mutations in *BRCA* genes are found in the minority of epithelial ovarian cancer cases, suggesting additional mechanisms involving other genes that predispose women to ovarian cancer. The location of the mutation within the *BRCA1* and *BRCA2* genes has been associated with different ovarian cancer risk (Rebbeck, 2015). Additionally, several common alleles, or alternate forms of a gene, have been found to modify ovarian cancer risk for *BRCA1* and *BRCA2* mutation carriers. These modifier genes alter the process by which information from a gene is used to synthesize a final gene product (gene expression) in another gene, which in turn causes a disease. They are hypothesized to act as low to moderate penetrance alleles that contribute to ovarian cancer risk. (Barnes and Antoniou, 2012; Ramus, 2008; Saed, 2017; Sellers, 2008). These modifiers consist of changes in the DNA called single-nucleotide variants (SNVs), and result in a point mutation in the gene. The mutation can result in a structurally altered protein that is functionally defective. Some of the affected proteins are oxidants, antioxidants, or otherwise involved in regulatory pathways involving cancer risk, as discussed below.

Lynch syndrome is another hereditary condition that increases the risk of ovarian cancer. It is caused by mutations that impair DNA mismatch repair, and the disease is inherited in an autosomal dominant manner similar to *BRCA* mutations. As in the case of *BRCA* mutations, due to incomplete penetrance inheriting a Lynch-associated mutation does not guarantee an individual will get cancer, but rather, that the risk of cancer will increase when exposed to a carcinogen.

Myriad Genetics was an early pioneer in the development of commercial genetic testing for *BRCA1* and *BRCA2* mutations and predicting risk for breast and ovarian cancer. As with all inherited traits, a positive family history is the strongest indicator of the presence of genetic risk alleles in an individual. Since the exact identity of those risk alleles and the magnitude of cancer risk remain unknown until testing is performed, early guidelines for testing were based on a positive family history. The availability of testing has increased and costs of testing have fallen. However, genetic testing remains a relatively rare practice in the general population. Since the

8

early 1990s, advanced molecular biological technologies have allowed for the connection to be made between specific genetic mutations and the resulting hereditary cancers. Because of the large number of individuals tested and the ability to trace their genetic inheritance, the genes involved in cancer development are well established. In the overall spectrum, there are additional variants and genes with minor involvement, but development is dependent upon specific and complex interactions that occur in rare situations, and it is extremely unlikely any would have impact of known mutations such as *BRCA1* or *BRCA2*.

V.   Response to Cellular Injury

As previously mentioned, from the moment of conception, the human body relies on continuous cell growth and development for normal health and function. Some tissues and cell types continually turn over. Our skin, blood cells, immune cells and the cells that line our digestive tract are examples where cells are continually growing and replacing older cells. In the case of an injury, a complex cascade of events begins which involves inflammation and culminates in the healing of the wound. During tissue injury, cell proliferation is enhanced while the tissue regenerates. After the healing is complete, proliferation and inflammation subside.

In contrast, proliferating cells that sustain DNA damage and/or mutagenic insult (for example, initiated cells) continue to proliferate in microenvironments rich in inflammatory cells and growth/survival factors that support their growth.   In a sense, tumors act as wounds that fail to heal (Dvorak, 1986). Recent studies have shown a link between inflammation associated with wound healing and ovarian cancer cell seeding (Jia, 2018). In addition to inflammation, the innate immune response plays a role in promoting cancer development and progression. These observations are generally accepted in the scientific literature (Coussens and Werb, 2002; Pardoll, 2002).

9

VI.   Inflammation

A.   The Role of Inflammation in Cancer - General

The functional relationship of cancer and inflammation was first described in the mid-1800s. Rudolf Virchow noted leucocytes in neoplastic tissues in 1863 and made a connection between inflammation and cancer (as cited in Balkwill and Mantovani, 2001). He suggested that the "lymphoreticular infiltrate" reflected the origin of cancer at sites of chronic inflammation. Research published over the last 20 years has provided further understanding of the inflammatory microenvironment of malignant tissues and validates Virchow's hypothesis. Furthermore, the links between cancer and inflammation now have quite strong implications for prevention and treatment. (Balkwill and Mantovani, 2001).

Macrophages are versatile immune-system cells that play a variety of roles in health and well-being. They act in tissues and free-floating cells in the blood that engulf and digest cellular debris, foreign substances, infectious microbes, cancer cells and anything that does not have the correct cell surface proteins to indicate a healthy cell to the body. They take various forms with various names throughout the body and have specialized tasks, including recruiting other immune cells like lymphocytes to sites of infection or acting as antigen presenting cells to T cells. Upon activation by contact with substances foreign to the body, macrophages release small proteins called cytokines. Generally speaking, macrophages can increase inflammation or decrease inflammation depending on the cytokines released.

Tumor-associated macrophages (TAM) are a major component of the infiltrate of most, if not all, tumors (Franklin and Li, 2016). TAM derive from circulating monocytic precursors, and are directed into the tumor by chemoattractant cytokines called chemokines. Many tumor cells also produce cytokines called colony-stimulating factors that prolong survival of TAM. When appropriately activated, TAM can kill tumor cells or elicit tissue destructive reactions on the vascular endothelium to disrupt blood supply to the tumor. However, TAM also produce growth and angiogenic factors as well as protease enzymes which degrade the extracellular matrix. Therefore, TAM can stimulate tumor-cell proliferation, promote angiogenesis, and favor invasion and metastasis (Mantovani, 1992b; Mantovani, 1997). Direct evidence for the importance of

10

protease production by TAM, neutrophils, and mast cells during experimental carcinogenesis was reported more than 15 years ago (Coussens, 2000). Since that time, the report by Coussens at el has been cited nearly 300 times by other studies.   This dual potential of TAM has been described in the literature as the "macrophage balance." (Liu and Cao, 2015; Mantovani, 1992a).

B.   The Role of Inflammation in Ovarian Cancer

Inflammation has also been shown to play a key role directly in epithelial ovarian cancer. This principle is generally accepted in the scientific community and very well reviewed in the scientific literature over the last decade, as the role of inflammation is common in many types of cancer. (Charbonneau, 2013; Kisielewski, 2013; Maccio and Madeddu, 2012; Mor , 2011; Pardoll, 2002; Pejovic and Nezhat, 2011; Shan and Liu, 2009). The literature reviews, as well as many direct studies, feature the immune system as being an important mediator of ovarian carcinogenesis via two models for its role in ovarian cancer: 1) chronic inflammation and 2) incessant ovulation.

1) <u>Chronic Inflammation:</u> The chronic inflammation model of carcinogenesis proposes that chronic exposures to external or endogenous triggers of immunity (such as known carcinogens) and the persistence of immune cells cause ovarian cancer.   These inflammatory triggers cause injury to surrounding epithelium, damage DNA through the release of reactive oxygen species (ROS), or produce cytokines that promote proliferation (Saed, 2017). One environmental exposure shown to induce inflammation in animal models and human lungs is talcum powder (Wehner, 1994). Composed primarily of magnesium silicate, talc has been linked to ovarian cancer risk in a number of studies (Ness, 2000; Mills, 2004; Merritt, 2008; Wu, 2009; Rosenblatt, 2011; Wu, 2015; Penninkilampi, 2018).

2) <u>Incessant Ovulation:</u> As stated in (Charbonneau, 2013), incessant ovulation results in damage due to rupturing of the ovulating follicle, which traumatizes the ovarian surface causing an immediate inflammatory response and wound repair. Repeating this process of damage and epithelial proliferation to repair the wound increases the risk of malignant transformation.   Epidemiologic studies beginning nearly 50 years ago have implicated increased number of ovulations as a risk factor for ovarian cancer (Mahdavi, 2006). In

11

contrast, decreased risk of (i.e., protection from) ovarian cancer has been associated with increased parity (Adami, 1994; Modan, 2001), oral contraceptive use (Narod , 1998), breast feeding (Jordan, 2012) and older age at first menses (Titus-Ernstoff, 2001). All of these protective factors impact the number of lifetime ovulations. One of these early studies from the late 1970's, which has been further substantiated by more recent investigations, found protective effects of "anovulatory time" by combining information on both increased oral contraceptive use and parity as well as age at first and last menses (Casagrande, 1979), supporting the theory of incessant ovulation as an underlying mechanism of carcinogenesis.

As a part of the inflammatory response, macrophages induce oxidative stress through production of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Normally, oxidants and antioxidants maintain a balance wherein the amount of ROS does not overwhelm the ability of the body, and antioxidants, to regulate them. Free radicals such as ROS and RNS are highly reactive and adversely alter DNA, proteins, and lipids (which comprise cell membranes) to promote tumor development and progression, and many cancers arise from sites that are subject to chronic irritation, infection, or inflammation. Cancer cells persist in a pro-oxidant state where there is excess production and generation of ROS that allows for tumor initiation, promotion and progression.

The association between exposure to pathogens and chronic inflammation in tumor promotion and progression is further support of the generally understood principle that chronic inflammation plays a key role in the development of ovarian cancer. Examples of inflammatory conditions that are associated with ovarian cancer include endometriosis and pelvic inflammatory disease. Evidence strongly suggests that endometriosis is a pelvic inflammatory condition (Agic, 2006), and that inflammation explains the association between endometriosis and epithelial ovarian cancer (Ness, 2000). Studies have found a relationship between pelvic inflammatory disease and ovarian cancer risk (Lin, 2011; Merritt, 2008). Moreover, the effect of non-steroidal anti-inflammatory drugs (NSAIDs) to reduce the risk of ovarian cancer provides additional support. The earlier studies with a focus on NSAIDs were preliminary and results were somewhat

inconsistent (Bonovas, 2005; Merritt, 2008), but a recent pooled analysis examining 12 case-control studies found aspirin could reduce ovarian cancer risk by 20%-34% (Trabert, 2014).

Additional studies illustrate the potential protective effects of anti-inflammatory agents, including from unexpected drugs such as metformin. As reviewed in Reid, 2017, evidence supports a role for the anti-diabetic agent, metformin, in the prevention and treatment of multiple cancers (Li, 2011). Studies reviewed include a case-control study including 1,611 incident ovarian cancer cases performed using the UK-based General Practice Research Database (Bodmer, 2011). Long-term use ($\geq$ 30 prescriptions) of metformin (and not sulfonylureas or insulin) was associated with a trend towards reduced risk with an odds ratio of 0.61. Though these results alone were not statistically significant, the reported observation that the anti-inflammatory agent, metformin, appears to decrease the risk of cancer, is additional evidence that inflammation is a primary mediator of ovarian cancer. (Irie, 2016).

Considering the well-established role that inflammation plays in cancer and the beneficial effects of anti-inflammatory compounds on cancer risk and progression, it is logical to examine the environmental factors that may directly lead to cancer or that may increase chronic inflammation and indirectly lead to cancer. The International Agency for Research on Cancer (IARC) has recognized for nearly thirty years that there is sufficient evidence to conclude human exposure to asbestos is a cause of ovarian cancer (IARC, 1987; IARC, 2012). Not surprisingly, human studies have reported asbestos fibers in ovaries (Heller, 1996; Langseth, 2007). Meta-analysis continues to support the conclusion that exposure to asbestos increases risk for ovarian cancer (Camargo et al., 2011).

C.   Talcum Powder Products

A number of studies have been performed to examine the role of talcum powder use in the development of ovarian cancers. A comprehensive and recent meta-analysis by Penninkilampi found an association between perineal talc use and ovarian cancer, with a greater association after a higher number of lifetime applications (Penninkilampi and Eslick, 2017). The Penninkilampi study identified 24 case–control (13,421 cases) and three cohort studies (890 cases). Observational studies involving at least 50 cases of ovarian cancer were eligible for inclusion. Penninkilampi

13

analyzed the association between ovarian cancer and any perineal talc use. Included studies reported specific types of ovarian cancer, long-term (>10 year) talc use total lifetime applications, frequency and use of talc while also using diaphragms or sanitary napkins.

The Penninkilampi study found a consistent association between perineal talc use and ovarian cancer. Variation in the magnitude of the effect was found when considering study design and ovarian cancer subtype. Any perineal talc use was associated with increased risk of ovarian cancer (OR=1.31, 95%CI 1.24-1.39). Greater than 3,600 lifetime applications (OR=1.42, 95%CI 1.25-1.61) was slightly more associated with ovarian cancer than less than 3,600 applications (OR=1.32, 95%CI 1.15- 1.50).

In addition to epidemiological evidence, an *in vitro* experiment by Buz'Zard and Lau reported an increase in ROS generation, increased cell proliferation and neoplastic transformation (conversion into cancerous cells) in human ovarian cells treated with talcum powder (Buz'Zard and Lau, 2007). They also found talcum powder treatment increased the number of reactive oxygen species produced by polymorphonuclear neutrophils, inflammatory cells whose role is to release large quantities of reactive oxygen species in response to a variety of harmful foreign stimuli. Additional studies have also shown the effects of talc on the immune response (Hamilton, 1984; Keskin, 2009; NTP, 1993).

Some studies have suggested that the link between ovarian cancer and talcum powder product use may be influenced by a number of genes (Belotte, 2015; Fletcher, 2018[a]; Gates, 2008; Shukla, 2009). Gates and colleagues found that women with certain genetic variants in glutathionine S-transferase M1 (GSTM1) and/or glutathionine S-transferase T1 (GSTT1) may have a higher risk of ovarian cancer associated with talc use (Gates, 2008). In a recently peer-reviewed and accepted abstract, Harper and Saed report a mechanism by which talc enhances the pro-oxidant state in normal (ovarian and tubal) and ovarian cancer cells, through induction of gene point mutations (corresponding to known specific single nucleotide polymorphisms - SNPs) in key oxidant enzymes, altering their activities (Harper and Saed, 2018).

In a more recent study, talcum powder increased mRNA levels of pro-oxidant enzymes in normal ovarian epithelial cells and ovarian cancer cell lines, while decreasing the mRNA levels of

14

antioxidant enzymes (Saed et al., 2017; Saed et al., 2018). A follow-up study reported in an abstract showed epithelial ovarian cancer cells treated with talc to demonstrate increased levels of CA-125 (Fletcher, 2018[b]). CA-125 is a biomarker that has been found to be elevated in patients with ovarian cancer and is currently FDA approved for disease monitoring in patients with epithelial ovarian cancer, as well as those with BRCA mutations or who are in another in high-risk group.

D.   Asbestos, Fibrous Talc, Heavy Metals and Fragrance Chemicals

In addition to the mineral talc, I have seen evidence that talcum powder products, including Johnson's Baby Powder and Shower to Shower, contain asbestos[2], and heavy metals[3] such as chromium, cobalt, and nickel. A 2017 study by Longo and Rigler on historic samples of Johnson & Johnson baby powder ranging in production date over a span of many years showed over one-half (17 of 30) of Johnson's talcum powder product samples contained asbestos (Longo and Rigler, 2017). Talc containing asbestiform fibers (fibrous talc) was found in 15 of the 30 samples. A 2018 study by Longo and Rigler reported the presence of fibrous anthophyllite in products tested from 1978 as well as fibrous talc in both (Longo and Rigler, 2018). Additionally, I have reviewed the expert report of Drs. Longo and Rigler reporting that 37 of 56 historical talcum powder samples contained asbestos and 41 of the 42 samples tested contained fibrous talc[4].

Asbestos has long been recognized as a well-known carcinogen and exposure can cause lung disease, mesothelioma, and cancers of the lung, larynx, and ovary (IARC 1987, 2012). It is established that asbestos exposure can result in macrophage activation, inflammation, generation of reactive oxygen and reactive nitrogen species, tissue injury, genotoxicity, and resistance to programmed cell death (Aust, 2011; Hein, 2007; IARC, 2012; Jaurand, 1997; Wang, 1987). One of the direct mechanisms is through interactions between internalized fibers and components of mitosis, resulting in chromosomal alterations and abnormalities (Hesterberg et al., 1986; Wang et al., 1987; Yegles et al., 1993). IARC has classified asbestos as a known human carcinogen (Group

---

[2] Ex. 28, Hopkins Dep. (Aug. 16 & 17, 2018; Oct. 17, 2018; and Nov. 5, 2018); Blount, 1991; Paoletti, 1984.
[3] Ex. 47, Julie Pier Dep. (Sept. 12 & 13, 2018).
[4] Expert Report of William E. Longo, PhD and Mark W. Rigler, PhD (Nov. 14, 2018).

1).   Human tumors resulting from asbestos exposure can be characterized by genetic and chromosomal alterations that lead to the inactivation of tumor-suppressor genes (IARC, 2012).

Talc not containing asbestiform fibers has been found by IARC to be a Group 2b or "possible" carcinogen (IARC, 2010). IARC has determined that fibrous talc or talc containing asbestiform fibers (talc occurring in a fibrous habit) is a carcinogen to humans (IARC, 2012).

Chromium and nickel are classified by IARC as Group 1, "carcinogenic to humans" (IARC, 2012). Cobalt is classified as Group 2B, "possibly carcinogenic to humans" (IARC, 2006). IARC defines possibly carcinogenic as "a positive association has been observed between exposure to the agent and cancer for which a causal interpretation has been considered by the Working Group to be credible, but chance, bias or confounding could not be ruled out with reasonable confidence." Established carcinogenic mechanisms of chromium include DNA damage, mutation, genomic instability, and cell transformation (IARC, 2009).   Similar mechanisms result from nickel exposure (IARC, 2012). Cobalt exposure has been to shown to cause increased production of reactive oxygen species and other inflammatory and proliferative changes (IARC, 2006).

I also reviewed Dr. Michael Crowley's report discussing the numerous fragrance chemicals added to talcum powder products. I am in agreement with Dr. Crowley's opinion that these chemicals contribute to the inflammatory properties, toxicity, and potential carcinogenicity of the products. The presence of these constituents as part of talcum powder products provides additional evidence of biological plausibility for talc and ovarian cancer.[5]

Carcinogenesis is a complex and dynamic process that occurs due to a combination of mutations, both genetic and acquired, in an individual along with other processes. Mutations arising from environmental sources have an additive, and possibly multiplicative effect toward ultimately causing carcinogenesis (Park, 2018; Vitonis, 2011; Wu, 2015). The presence of asbestos, nickel, and chromium, known carcinogens, in talcum powder products provides further support for the conclusion that talcum powder causes chronic inflammation.

---

[5] Expert Report of Michael Crowley, PhD (Nov. 12, 2018).

Based on these observations and lines of evidence, it is my opinion that talcum powder causes inflammation which initiates a biological response that includes oxidative stress, cell proliferation, inhibition of apoptosis, and genetic mutations which result in cancer development and progression. This process explains the biologically plausible mechanism for talcum powder products causing ovarian cancer.

VII.   Conclusion

Based on my background, training, education, and experience as a geneticist assessing and weighing the totality of scientific evidence, my opinions may be summarized as follows:

1. Genetic mutations can be inherited or acquired. Both types are associated with cancer, including ovarian cancer.
2. Talcum powder products cause chronic inflammation.
3. Talcum powder product-induced inflammation causes damage to the DNA, genetic mutation, genomic instability, and cell transformation.
4. The properties of talcum powder products as inflammatory agents and the role of inflammation in triggering oxidative stress, activating cytokines, cell proliferation, DNA damage, and genetic mutations (such as SNVs) provide a biologically plausible mechanism for the carcinogenicity of talcum powder products.
5. Internalization of asbestiform fibers (including fibrous talc), cause DNA damage which provides a biologically plausible mechanism for the carcinogenicity of talcum powder products.
6. The presence of an inherited gene mutation, such as *BRCA1* or *BRCA2*, indicates a woman has an increased risk of ovarian cancer, but does not necessarily mean she will develop ovarian cancer.
7. Women with inherited gene mutations, such as *BRCA*, are at least as susceptible to other carcinogens as women without inherited gene mutations.

17

I reserve the right to supplement, revise, or amend this report should additional materials, including testimony, become available.

Literature Cited

Adami, H.O., Hsieh, C.C., Lambe, M., Trichopoulos, D., Leon, D., Persson, I., Ekbom, A., and Janson, P.O. (1994). Parity, age at first childbirth, and risk of ovarian cancer. Lancet *344*, 1250-1254.

Agic, A., Xu, H., Finas, D., Banz, C., Diedrich, K., and Hornung, D. (2006). Is endometriosis associated with systemic subclinical inflammation? Gynecol Obstet Invest *62*, 139-147.

Aust, A.E., Cook, P.M., and Dodson, R.F. Morphological and chemical mechanisms of elongated mineral particle toxicities. J Toxicol and Environ Health, Part B. (2011) 14:40-75.

Balkwill, F., and Mantovani, A. (2001). Inflammation and cancer: back to Virchow? Lancet *357*, 539-545.

Banerjee, S., and Kaye, S.B. (2013). New strategies in the treatment of ovarian cancer: current clinical perspectives and future potential. Clin Cancer Res *19*, 961-968.

Barnes, D.R., and Antoniou, A.C. (2012). Unravelling modifiers of breast and ovarian cancer risk for BRCA1 and BRCA2 mutation carriers: update on genetic modifiers. J Intern Med *271*, 331-343.

Belotte, J., Fletcher, N.M., Saed, M.G., Abusamaan, M.S., Dyson, G, Diamond, M.P., Saed, G.M. (2015) A single nucleotide polymorphism in catalase is strongly associated with ovarian cancer survival. PLoS ONE 10(8): e0135739. Doi: 10.1371/journal.pone.0135739.

Berek, J.S., Crum, C., and Friedlander, M. (2012). Cancer of the ovary, fallopian tube, and peritoneum. Int J Gynaecol Obstet *119 Suppl 2*, S118-129.

Blount, A.M. (1991). Amphibole content of cosmetic and pharmaceutical talcs. Environ Health Perspect *94*, 225-230.

Bodmer, M., Becker, C., Meier, C., Jick, S.S., and Meier, C.R. (2011). Use of metformin and the risk of ovarian cancer: a case-control analysis. Gynecol Oncol *123*, 200-204.

Bonovas, S., Filioussi, K., and Sitaras, N.M. (2005). Do nonsteroidal anti-inflammatory drugs affect the risk of developing ovarian cancer? A meta-analysis. Br J Clin Pharmacol *60*, 194-203.

Bowtell, D.D. (2010). The genesis and evolution of high-grade serous ovarian cancer. Nat Rev Cancer *10*, 803-808.

Buz'Zard, Amber R., and Benjamin H. S. Lau. "Pycnogenol® Reduces Talc-Induced Neoplastic Transformation in Human Ovarian Cell Cultures." *Phytotherapy Research* 21, No. 6 (June 2007): 579–86.

Camargo, M.C., Stayner, L.T., Straif, K., Reina, M., Al-Alem, U., Demers, P.A., and Landrigan, P.J. (2011). Occupational exposure to asbestos and ovarian cancer: a meta-analysis. Environ Health Perspect *119*, 1211-1217.

Casagrande, J.T., Louie, E.W., Pike, M.C., Roy, S., Ross, R.K., and Henderson, B.E. (1979). "Incessant ovulation" and ovarian cancer. Lancet *2*, 170-173.

Catalani, S. (2009). [Lancet-Oncology--a summary of the review on IARC carcinogens: metallic elements, dusts and fibers]. G Ital Med Lav Ergon *31*, 182-183.

Chan, J.K., Cheung, M.K., Husain, A., Teng, N.N., West, D., Whittemore, A.S., Berek, J.S., and Osann, K. (2006). Patterns and progress in ovarian cancer over 14 years. Obstet Gynecol *108*, 521-528.

Charbonneau, B., Goode, E.L., Kalli, K.R., Knutson, K.L., and Derycke, M.S. (2013). The immune system in the pathogenesis of ovarian cancer. Crit Rev Immunol *33*, 137-164.

Coussens, L.M., Tinkle, C.L., Hanahan, D., and Werb, Z. (2000). MMP-9 supplied by bone marrow-derived cells contributes to skin carcinogenesis. Cell *103*, 481-490.

Coussens, L.M., and Werb, Z. (2002). Inflammation and cancer. Nature *420*, 860-867.

Dvorak, H.F. (1986). Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. N Engl J Med *315*, 1650-1659.

Fletcher, N.M., Belotte, J., Saed, M.G., Memaj, I., Diamond, M.P., Morris, R.T., Saed, G.M. Specific point mutations in key redox enzymes are associated with chemoresistance in epithelial ovarian cancer. Free Radical Biology and Medicine (2017) 102:122-132.

Fletcher[a], N.M., Memaj, I., and Saed, G.M. (2018). Talcum Powder Enhances Oxidative Stress in Ovarian Cancer Cells. Paper presented at: REPRODUCTIVE SCIENCES (SAGE PUBLICATIONS INC 2455 TELLER RD, THOUSAND OAKS, CA 91320 USA).

Fletcher[b], N.M., Saed, G.M. (2018). Talcum Powder enhances cancer antigen 125 levels in ovarian cancer cells and in normal ovarian epithelial cells. LB-044. Poster presented at: Society for Reproductive Investigation. 65th annual meeting. San Diego, CA.

Franklin, R.A., and Li, M.O. (2016). Ontogeny of Tumor-associated Macrophages and Its Implication in Cancer Regulation. Trends Cancer *2*, 20-34.

Gates, M.A., Tworoger, S.S., Terry, K.L., Titus-Ernstoff, L., Rosner, B., De Vivo, I., Cramer, D.W., and Hankinson, S.E. (2008). Talc use, variants of the GSTM1, GSTT1, and NAT2 genes, and risk of epithelial ovarian cancer. Cancer Epidemiol Biomarkers Prev *17*, 2436-2444.

Hamilton, T.C., Fox, H., Buckley, C.H., Henderson, W.J., and Griffiths, K. Effects of talc on the rat ovary. Br. J. Exp. Path. (1984) *65*: 101-106.

Harper, A.K., Saed, G.M. Talc induces a pro-oxidant state in normal and ovarian cancer cells through gene point mutations in key redox enzymes. Accepted for presentation at SGO meeting 2019. [In Press].

Hein, M.J., Stayner, L.T., Lehman, E., Dement, J.M. Follow-up study of chrysotile textile workers: cohort mortality and exposure-response. Occup Environ Med (2007) 64:616-625.

Heller, D.S., Gordon, R.E., Westhoff, C., and Gerber, S. (1996). Asbestos exposure and ovarian fiber burden. Am J Ind Med *29*, 435-439.

Hesterberg, T.W., Butterick, C.J., Oshimura, M., Brody, A.R., and Barrett, J.C. (1986). Role of phagocytosis in Syrian hamster cell transformation and cytogenetic effects induced by asbestos and short and long glass fibers. Cancer Res *46*, 5795-5802.

International Agency for Research on Cancer (IARC), "Silica and Some Silicates", IARC Monograph No. 42 (1987).

International Agency for Research on Cancer (IARC), "Cobalt in Hard Metals and Cobalt Sulfate, Gallium Arsenide, Indium Phosphide and Vanadium Pentoxide," IARC Monograph No. 86 (2006).

International Agency for Research on Cancer (IARC), "Arsenic, Metals, Fibres, and Dusts," IARC Monograph No. 100C (2009).

International Agency for Research on Cancer (IARC), "Carbon Black, Titanium Dioxide, and Talc", IARC Monograph No. 93 (2010).

International Agency for Research on Cancer (IARC), "Arsenic, Metals, Fibres, and Dusts", IARC Monograph No. 100C (2012).

Irie, H., Banno, K., Yanokura, M., Iida, M., Adachi, M., Nakamura, K., Umene, K., Nogami, Y., Masuda, K., Kobayashi, Y*., et al.* (2016). Metformin: A candidate for the treatment of gynecological tumors based on drug repositioning. Oncol Lett *11*, 1287-1293.

Jayson, G.C., Kohn, E.C., Kitchener, H.C., and Ledermann, J.A. (2014). Ovarian cancer. Lancet *384*, 1376-1388.

Jaurand, M.C. Mechanisms of fiber-induced genotoxicity. Environ Health Perspect (1997) 105(Suppl 5):1073-1084.

Jia, D., Nagaoka, Y., Katsumata, M., and Orsulic, S. (2018). Inflammation is a key contributor to ovarian cancer cell seeding. Sci Rep *8*, 12394.

Jordan, S.J., Cushing-Haugen, K.L., Wicklund, K.G., Doherty, J.A., and Rossing, M.A. (2012). Breast-feeding and risk of epithelial ovarian cancer. Cancer Causes Control *23*, 919-927.

Keskin, N., Teksen, Y.A., Ongun, E.G., Ozay, Y., Saygili, H. Does long-term talc exposure have a carcinogenic effect on the female genital system of rats? An experimental pilot study. Arch Gynecol Obstet. (2009). 280:925-931.

Kisielewski, R., Tolwinska, A., Mazurek, A., and Laudanski, P. (2013). Inflammation and ovarian cancer--current views. Ginekol Pol *84*, 293-297.

Langseth, H., Johansen, B.V., Nesland, J.M., and Kjaerheim, K. (2007). Asbestos fibers in ovarian tissue from Norwegian pulp and paper workers. Int J Gynecol Cancer *17*, 44-49.

Levanon, K., Crum, C., Drapkin, R. New insights into the pathogenesis of serous ovarian cancer and its clinical impact. J Clin Oncol. (2008). Nov 10;*26*(32):5284-93.

Li, D. (2011). Metformin as an antitumor agent in cancer prevention and treatment. J Diabetes *3*, 320-327.

Lin, H.W., Tu, Y.Y., Lin, S.Y., Su, W.J., Lin, W.L., Lin, W.Z., Wu, S.C., and Lai, Y.L. (2011). Risk of ovarian cancer in women with pelvic inflammatory disease: a population-based study. Lancet Oncol *12*, 900-904.

Liu, Y., and Cao, X. (2015). The origin and function of tumor-associated macrophages. Cell Mol Immunol *12*, 1-4.

Longo, W., and Rigler, M. (2017). Analysis of Johnson & Johnson Baby Powder & Valiant Shower to Shower Talc Products for Amphibole (Tremolite) Asbestos (Materials Analytical Services, LLC).

Longo, W., and Rigler, M. (2018). TEM Analysis of Historical 1978 Johnson's Baby Powder Sample for Amphibole Asbestos (Materials Analytical Services, LLC).

Mabuchi, S., Kawase, C., Altomare, D.A., Morishige, K., Sawada, K., Hayashi, M., Tsujimoto, M., Yamoto, M., Klein-Szanto, A.J., Schilder, R.J.*, et al.* (2009). mTOR is a promising therapeutic target both in cisplatin-sensitive and cisplatin-resistant clear cell carcinoma of the ovary. Clin Cancer Res *15*, 5404-5413.

Maccio, A., and Madeddu, C. (2012). Inflammation and ovarian cancer. Cytokine *58*, 133-147.

Mahdavi, A., Pejovic, T., and Nezhat, F. (2006). Induction of ovulation and ovarian cancer: a critical review of the literature. Fertil Steril *85*, 819-826.

Mantovani, A., Bottazzi, B., Colotta, F., Sozzani, S., and Ruco, L. (1992a). The origin and function of tumor-associated macrophages. Immunol Today *13*, 265-270.

Mantovani, A., Bussolino, F., and Dejana, E. (1992b). Cytokine regulation of endothelial cell function. FASEB J *6*, 2591-2599.

Mantovani, A., Bussolino, F., and Introna, M. (1997). Cytokine regulation of endothelial cell function: from molecular level to the bedside. Immunol Today *18*, 231-240.

21

Merritt, M.A., Green, A.C., Nagle, C.M., Webb, P.M., Australian Cancer, S., and Australian Ovarian Cancer Study, G. (2008). Talcum powder, chronic pelvic inflammation and NSAIDs in relation to risk of epithelial ovarian cancer. Int J Cancer *122*, 170-176.

Mills, P.K., Riordan, D.G., Cress, R.D., and Young, H.A. (2004). Perineal talc exposure and epithelial ovarian cancer risk in the Central Valley of California. Int J Cancer *112*, 458-464.

Modan, B., Hartge, P., Hirsh-Yechezkel, G., Chetrit, A., Lubin, F., Beller, U., Ben-Baruch, G., Fishman, A., Menczer, J., Struewing, J.P.*, et al.* (2001). Parity, oral contraceptives, and the risk of ovarian cancer among carriers and noncarriers of a BRCA1 or BRCA2 mutation. N Engl J Med *345*, 235-240.

Mor, G., Yin, G., Chefetz, I., Yang, Y., and Alvero, A. (2011). Ovarian cancer stem cells and inflammation. Cancer Biol Ther *11*, 708-713.

Narod, S.A., Risch, H., Moslehi, R., Dorum, A., Neuhausen, S., Olsson, H., Provencher, D., Radice, P., Evans, G., Bishop, S.*, et al.* (1998). Oral contraceptives and the risk of hereditary ovarian cancer. Hereditary Ovarian Cancer Clinical Study Group. N Engl J Med *339*, 424-428.

National Toxicology Program. Toxicology and carcinogenesis studies of talc (CAS No. 14807-96-6) in F344/N rats and B6C3F$_1$ mice (Inhalation studies); 1993. Report No. NTP TR 421; NIH Publication No. 93-3152.

Ness, Roberta B., Jeane Ann Grisso, Carrie Cottreau, Jennifer Klapper, Ron Vergona, James E. Wheeler, Mark Morgan, and James J. Schlesselman. "Factors Related to Inflammation of the Ovarian Epithelium and Risk of Ovarian Cancer." *Epidemiology 11*, No. 2 (2000): 111–17.

Nunes, S.C., and Serpa, J. (2018). Glutathione in Ovarian Cancer: A Double-Edged Sword. Int J Mol Sci *19*.

Paluch-Shimon, S., Cardoso, F., Sessa, C., Balmana, J., Cardoso, M.J., Gilbert, F., Senkus, E., and Committee, E.G. (2016). Prevention and screening in BRCA mutation carriers and other breast/ovarian hereditary cancer syndromes: ESMO Clinical Practice Guidelines for cancer prevention and screening. Ann Oncol *27*, v103-v110.

Paoletti, L., Caiazza, S., Donelli, G., and Pocchiari, F. (1984). Evaluation by electron microscopy techniques of asbestos contamination in industrial, cosmetic, and pharmaceutical talcs. Regul Toxicol Pharmacol *4*, 222-235.

Pardoll, D.M. (2002). Spinning molecular immunology into successful immunotherapy. Nat Rev Immunol *2*, 227-238.

Park, H.K., Schildkraut, J.M., Alberg, A.J., Bandera, E.V., Barnholtz-Sloan, J.S., Bondy, M., Crankshaw, S., Funkhouser, E., Moorman, P.G., Peters, E.S.*, et al.* (2018). Benign gynecologic conditions are associated with ovarian cancer risk in African-American women: a case-control study. Cancer Causes Control *29*, 1081-1091.

Pejovic, T., and Nezhat, F. (2011). Missing link: inflammation and ovarian cancer. Lancet Oncol *12*, 833-834.

Penninkilampi, Ross and Guy D. Eslick. "Perineal Talc Use and Ovarian Cancer: A Systematic Review and Meta-Analysis." *Epidemiology* 29, No. 1 (January 2018): 41-49.

Ramus, S.J., Vierkant, R.A., Johnatty, S.E., Pike, M.C., Van Den Berg, D.J., Wu, A.H., Pearce, C.L., Menon, U., Gentry-Maharaj, A., Gayther, S.A.*, et al.* (2008). Consortium analysis of 7 candidate SNPs for ovarian cancer. Int J Cancer *123*, 380-388.

Rebbeck, T.R., Mitra, N, Wan, F., Sinilnikova, O.M., Healey, S., McGuffog, L., Mazoyer, S., Chenevix-Trench, G., Easton, D.F., Antoniou, A.C., Nathanson, K.L., the CIMBA Consortium. Association fo type and location of BRCA1 and BRCA2 mutations with risk of breast and ovarian cancer. JAMA. (2015). April 7; 313(13):1347-1361.

Reid, B.M., Permuth, J.B., Sellers, T.A. Epidemiology of ovarian cancer: a review. Cancer Med Biol. (February 2017) Vol *14*, No 1.

Rosenblatt, K.A., Weiss, N.S., Cushing-Haugen, K.L., Wicklund, K.G., and Rossing, M.A. (2011). Genital powder exposure and the risk of epithelial ovarian cancer. Cancer Causes Control *22*, 737-742.

Saed, G.M., Diamond, M.P., and Fletcher, N.M. (2017). Updates of the role of oxidative stress in the pathogenesis of ovarian cancer. Gynecol Oncol *145*, 595-602.

Saed, G.M., Morris, R.T., and Fletcher, N.M. (2018). New Insights into the Pathogenesis of Ovarian Cancer: Oxidative Stress. In Ovarian Cancer-From Pathogenesis to Treatment (IntechOpen).

Sellers, T.A., Huang, Y., Cunningham, J., Goode, E.L., Sutphen, R., Vierkant, R.A., Kelemen, L.E., Fredericksen, Z.S., Liebow, M., Pankratz, V.S.*, et al.* (2008). Association of single nucleotide polymorphisms in glycosylation genes with risk of epithelial ovarian cancer. Cancer Epidemiol Biomarkers Prev *17*, 397-404.

Shan, W., and Liu, J. (2009). Inflammation: a hidden path to breaking the spell of ovarian cancer. Cell Cycle *8*, 3107-3111.

Shen, H., Fridley, B.L., Song, H., Lawrenson, K., Cunningham, J.M., Ramus, S.J., Cicek, M.S., Tyrer, J., Stram, D., Larson, M.C.*, et al.* (2013). Epigenetic analysis leads to identification of HNF1B as a subtype-specific susceptibility gene for ovarian cancer. Nat Commun *4*, 1628.

Shukla, A., MacPherson, M.B., Hillegass, J., Ramos-Nino, M.E., Alexeeva, V., Vacek, P.M., Bond, J.P., Pass, H.I., Steele, C., Mossman, B.T. Alterations in gene expression in human mesothelial cells correlate with mineral pathogenicity. Am J Respir Cell Mol Biol. (2009). 41:114-123.

Siegel, R.L., Miller, K.D., and Jemal, A. (2015). Cancer statistics, 2015. CA Cancer J Clin *65*, 5-29.

Titus-Ernstoff, L., Perez, K., Cramer, D.W., Harlow, B.L., Baron, J.A., and Greenberg, E.R. (2001). Menstrual and reproductive factors in relation to ovarian cancer risk. Br J Cancer *84*, 714-721.

Torre, L.A., Bray, F., Siegel, R.L., Ferlay, J., Lortet-Tieulent, J., and Jemal, A. (2015). Global cancer statistics, 2012. CA Cancer J Clin *65*, 87-108.

Trabert, B., Ness, R.B., Lo-Ciganic, W.H., Murphy, M.A., Goode, E.L., Poole, E.M., Brinton, L.A., Webb, P.M., Nagle, C.M., Jordan, S.J.*, et al.* (2014). Aspirin, nonaspirin nonsteroidal anti-inflammatory drug, and acetaminophen use and risk of invasive epithelial ovarian cancer: a pooled analysis in the Ovarian Cancer Association Consortium. J Natl Cancer Inst *106*, djt431.

Vitonis, A.F., Titus-Ernstoff, L., and Cramer, D.W. (2011). Assessing ovarian cancer risk when considering elective oophorectomy at the time of hysterectomy. Obstet Gynecol *117*, 1042-1050.

Wang, N.S., Jaurand, M.C., Magne, L., Kheuang, L., Pinchon, M.C., and Bignon, J. (1987). The interactions between asbestos fibers and metaphase chromosomes of rat pleural mesothelial cells in culture. A scanning and transmission electron microscopic study. Am J Pathol *126*, 343-349.

Wehner, A.P. (1994). Biological effects of cosmetic talc. Food Chem Toxicol *32*, 1173-1184.

Wu, A.H., Pearce, C.L., Tseng, C.C., and Pike, M.C. (2015). African Americans and Hispanics Remain at Lower Risk of Ovarian Cancer Than Non-Hispanic Whites after Considering Nongenetic Risk Factors and Oophorectomy Rates. Cancer Epidemiol Biomarkers Prev *24*, 1094-1100.

Wu, A.H., Pearce, C.L., Tseng, C.C., Templeman, C., and Pike, M.C. (2009). Markers of inflammation and risk of ovarian cancer in Los Angeles County. Int J Cancer *124*, 1409-1415.

Yegles, M., Saint-Etienne, L., Renier, A., Janson, X., and Jaurand, M.C. (1993). Induction of metaphase and anaphase/telophase abnormalities by asbestos fibers in rat pleural mesothelial cells in vitro. Am J Respir Cell Mol Biol *9*, 186-191.

Exhibit A

Shawn Edward Levy   *Curriculum Vitae*

Faculty Investigator   phone: 256.327.5267
HudsonAlpha Institute for Biotechnology   fax: 256.327.9898
601 Genome Way   slevy@hudsonalpha.org
Huntsville, AL 35806
DUNS number: 363616223

Personal Statement

My group has been utilizing high performance genotyping and sequencing technologies for the past 15 years supporting a vast diversity of projects from plant and animal phylogenetic studies to translational and clinical based projects. We have several publications detailing our successes using variety of genomic technologies as well as in the field of bioinformatics research. As a post-doctoral fellow at Emory University I developed the first microarray designed to interrogate mitochondrial gene function. Upon joining the faculty at Vanderbilt University, I was responsible for the founding and development of the Vanderbilt Microarray Shared Resource (VMSR). From 2000 to 2009, the VMSR became an internationally recognized facility supporting a wide variety of genomic technologies from SNP profiling to gene expression analysis to next-generation sequencing. I joined the faculty of the HudsonAlpha Institute for Biotechnology in 2009 to develop the Genomic Services Laboratory (GSL). Since 2009 the GSL has supported more than 1,000 principle investigators from around the world, allowing me to collaborate and participate in a broad range of genomics projects with a particular focus on applying a diversity of genomic methods to understand complex conditions. We have had a particular focus on childhood and adult cancer as well as rare disease and degenerative diseases. Together, these efforts have resulted in more than 140 peer-reviewed publications of which I am an author or co-author. More than 150 additional publications that have included data from our laboratory as a service provider have also been published since 2009. Many of these publications involve translational research or describe the genetic underpinnings of rare or complex human disease. The diversity of projects and investigators we have worked with over the last 15 years have provided a dynamic and amazing experience to evolve our own research and technology development efforts.

Contributions to Science

The following five sections provide highlights to areas where my work has contributed to areas of science. Example publications are provided with each section and a full bibliography is provided at the end of the CV.

1. My scientific career has been a somewhat atypical in that I have spent the last 15 years focusing on the development and application of genomic and bioinformatic technologies and methods to support scientific investigation in a number of areas. While there have been substantial areas of focus, my laboratory does not operate under a single or specific biological area or hypothesis. Instead, we examine ways to improve the resolution and quality of results to answer complex questions, regardless of biological relationship. The publications below are examples of contributions to technical projects or large consortium projects with goals in the evaluation or improvement of techniques or technologies.

    a. Statnikov A, Aliferis, C, Tsamardinos, I, Hardin, D, and Levy, S. A comprehensive evaluation of multicategory classification methods for microarray gene expression cancer diagnosis. **Bioinformatics**, 2005. 21(5), p. 631-643. PMID:15374862.

b. The MicroArray Quality Control Consortium. The MicroArray Quality Control (MAQC) project shows inter- and intraplatform reproducibility of gene expression measurements. **Nature Biotechnology**, 2006. 24(9), p. 1151-1161. PMID:16964229.

c. The ENCODE Project Consortium. An integrated encyclopedia of DNA elements in the human genome. **Nature**. 2012. 489, 57-74. PMID: 22955616 PMCID: PMC3439153

d. The Sequence Quality Control (SEQC) Consortium. A comprehensive assessment of RNA-seq accuracy, reproducibility and information content by the Sequence Quality Control consortium. **Nature Biotechnology**. 2014. 32 (9), 915-925. PMID:25150835; PMCID:4167418.

2. One area of early focus of my career was the development and analysis of mouse models for mitochondrial disease, including the knock out of the Adenine Nucleotide Translocase 2 (Ant2) gene leading to a more complete understanding of the permeability transition. This work also discovered methods to alter the mitochondrial DNA in stem cells and supported the first mitochondrial DNA transfers by stem cells.

a. Levy SE, Waymire, KG, Kim, YL, MacGregor, GR, and Wallace, DC, Transfer of chloramphenicol-resistant mitochondrial DNA into the chimeric mouse*.* **Transgenic Research**. 1999. 8(2), p. 137-145. PMID:10481313.

b. Sligh JE, Levy SE, Waymire KG, Allard P, Dillehay DL, Nusinowitz S, Heckenlively JR, MacGregor GR, and Wallace DC. Maternal germ-line transmission of mutant mtDNAs from embryonic stem cell-derived chimeric mice. **Proc. of the Nat. Acad. of Sciences USA.** 2000. 97(26), p. 14461-14466. PMID:11106380; PMCID:18941.

c. Kokoszka JE, Waymire, KG, Levy, SE, Sligh, JE, Cal, JY, Jones, DP, MacGregor, GR, and Wallace, DC, The ADP/ATP translocator is not essential for the mitochondrial permeability transition pore*.* **Nature**, 2004. 427(6973),p. 461-465. PMID:14749836.

d. Picard M, Zhang J, Hanecock S, Derbeneva O, Golhar R, Golik P, O'Hearn S, Levy SE, Potluri P, Lvova M, Davila A, Lin CS, Perin JC, Rappaport EF, Hakonarson H, Trounce I, Procaccio V, and Wallace DC. Progressive increase in mtDNA 3243A>G heteroplasmy results in abrupt transcriptional remodeling. **Proc. of the Nat. Acad. of Sciences USA**. 2014. 111(38), E4033-E4042*.* PMID:25192935; PMCID:4183335.

3. A long-standing area of research interest is the genomic analysis of cancer, both childhood and adult. These efforts have included population-based studies and more directed research in specific cancer biology. These efforts have examined many cancer types including breast, lung, colon, and myeloid cancer.

a. Smith JJ, Deane, NG, Wu, F, Merchant, NB, Zhang, B, Jiang, A, Lu, P, Johnson, JC, Schmidt, C, Edwards, CM, Eschrich, S, Kis, C, Levy, S, Washington, MK, Heslin, MJ, Coffey, RJ, Yeatman, TJ, Shyr, Y, and Beauchamp, RD, Experimentally Derived Metastasis Gene Expression Profile Predicts Recurrence and Death in Patients With Colon Cancer*.* **Gastroenterology**, 2009. PMID: 19914252 PMCID: PMC3388775.

b. Powell AE, Wang Y, Li Y, Poulin EJ, Means AL, Washington MK, Higginbotham JN, Juchheim A, Prasad N, Levy SE, Guo Y, Shyr Y, Aronow BJ, Haigis KM, Franklin JL, and Coffey RJ. Lrig1, a pan-ErbB negative regulator, marks intestinal stem cells and acts as a tumor suppressor. **Cell**. 2012. 149(1), 146-158. PMID: 22464327 PMCID: PMC3563328.

c. McDaniel JM, Varley KE, Gertz J, Savic DS, Roberts BS, Bailey SK, Shevde LA, Ramaker RC, Lasseigne BN, Kirby MK, Newberry KM, Partridge EC, Jones AL, Boone B, Levy SE, Oliver PG, Sexton KC, Grizzle WE, Forero A, Buchsbaum DJ, Cooper SJ, Myers RM. Genomic regulation of invasion by STAT3 in triple negative breast cancer. **Oncotarget**. 2017;8(5):8226-38. doi: 10.18632/oncotarget.14153. PubMed PMID: 28030809; PMCID: PMC5352396.

d. McKinney M, Moffitt AB, Gaulard P, Travert M, De Leval L, Nicolae A, Raffeld M, Jaffe ES, Pittaluga S, Xi L, Heavican T, Iqbal J, Belhadj K, Delfau-Larue MH, Fataccioli V, Czader MB, Lossos IS, Chapman-Fredricks JR, Richards KL, Fedoriw Y, Ondrejka SL, Hsi ED, Low L, Weisenburger D, Chan WC, Mehta-Shah N, Horwitz S, Bernal-Mizrachi L, Flowers CR, Beaven AW, Parihar M, Baseggio L, Parrens M, Moreau A, Sujobert P, Pilichowska M, Evens AM, Chadburn A, Au-Yeung RK, Srivastava G, Choi WW, Goodlad JR, Aurer I, Basic-Kinda S, Gascoyne RD, Davis NS, Li G, Zhang J, Rajagopalan D, Reddy A, Love C, Levy S, Zhuang Y, Datta J, Dunson DB, Dave SS. The Genetic Basis of Hepatosplenic T-cell Lymphoma. **Cancer Discov**. 2017;7(4):369-79. doi: 10.1158/2159-8290.CD-16-0330. PubMed PMID: 28122867; PMCID: PMC5402251.

4. My laboratory has had the opportunity to collaborate with a number of outstanding investigators in the genetics analysis of complex neurological conditions, including autism, schizophrenia and bipolar disorders as well as ALS. We contributed significantly to the discovery of the association of de-novo rather than Mendelian mutations in these conditions, particularly in schizophrenia.

   a. Xu B, Roos JL, Dexheimer P, Boone B, Plummer B, Levy S, Gogos JA, Karayiorgou M. Exome sequencing supports a de novo mutational paradigm for schizophrenia. **Nature Genetics**. 2011. 43(9), 864-868. PMID: 21822266. PMCID: PMC3196550.

   b. Neale BM, Kou Y, Liu L, Ma'ayan A, Samocha KE, Sabo A, Lin CF, Stevens C, Wang LS, Makarov V, Polak P, Yoon S, Maguire J, Crawford EL, Campbell NG, Geller ET, Valladares O, Shafer C, Liu H, Zhao T, Cai G, Lihm J, Dannenfelser R, Jabado O, Peralta Z, Nagaswamy U, Reid JG, Newsham I, Wu Y, Lewis L, Han Y, Muzny D, Voight BF, Lim E, Rossin E, Kirby A, Flannick J, Fromer M, Shakir K, Fennell T, Garimella K, Boyko C, Gabriel S, dePristo M, Wimbish JR, Boone BE, Levy SE, Betancur C, Sunyaev S, Boerwinkle E, Buxbaum JD, Cook EH, Devlin B, Gibbs R, Roeder K, Schellenberg GD, Sutcliffe JS, and Daly MJ. Patterns and rates of exonic de novo mutations in autism spectrum disorders. **Nature**. 2012. 485(7397), 242-245. PMID: 22495311 PMCID:PMC3613847.

   c. Xu B, Ionita-Laza I, Roos JL, Boone B, Woodrick S, Sun Y, Levy S, Gogos JA, and Karayiorgou M. De novo gene mutations highlight patterns of genetic and neural complexity in schizophrenia. **Nature Genetics**. 2012. 44(12), 1365-1369. PMID: 23042115 PMCID: PMC3556813.

   d. Cirulli, ET, Lasseigne, BN, Petrovski, S, Sapp, PC, Dion, PA, Leblond, CS, Couthouis, J, Lu, Y-F, Wang, Q, Krueger, BJ, Ren, Z, Keebler, J, Han, Y, Levy, SE, Boone, BE, Wimbish, JR, Waite, LL, Jones, AL, Carulli, JP, Day-Williams, AG, Staropoli, JF, Xin, WW, Chesi, A, Raphael, AR, McKenna-Yasek, D, Cady, J, Vianney de Jong, JMB, Kenna, KP, Smith, BN, Topp, S, Miller, J, Gkazi, A, Consortium, FS, Al-Chalabi, A, van den Berg, LH, Veldink, J, Silani, V, Ticozzi, N, Shaw, CE, Baloh, RH, Appel, S, Simpson, E, Lagier-Tourenne, C, Pulst, SM, Gibson, S, Trojanowski, JQ, Elman, L, McCluskey, L, Grossman, M, Shneider, NA, Chung, WK, Ravits, JM, Glass, JD, Sims, KB, Van Deerlin, VM, Maniatis, T, Hayes, SD, Ordureau, A, Swarup, S, Landers, J, Baas, F, Allen, AS, Bedlack, RS, Harper, JW, Gitler, AD, Rouleau, GA, Brown, R, Harms, MB, Cooper, GM, Harris, T, Myers, RM, Goldstein, DB. Exome sequencing in amyotrophic lateral sclerosis identifies risk genes and pathways. **Science**. 2015. Feb 19. pii: aaa3650. [Epub ahead of print] PubMed PMID: 25700176.

5. My laboratory has played a significant role in the discovery of the causative mutations of a number of rare but significant human diseases, particularly in the field of pediatric nephrology in collaboration with Friedhelm Hildebrandt at Harvard University. These studies applied genomic technologies to better characterize and in some cases diagnose or discover the causative mutation for severe phenotypes or disease.

   a. Otto EA, Hurd TW, Airik R, Chaki M, Zhou W, Stoetzel C, Patil SB, Levy S, Ghosh AK, Murga-Zamalloa CA, van Reeuwijk J, Letteboer SJF, Sang L, Giles RH, Liu Q, Coene KLM, Estrada-

Page 3 of 34

Cuzcano A, Collin RWJ, McLaughlin HM, Held S, Kasanuki JM, Ramaswami G, Conte J, Lopez I, Washburn J, MacDonald J, Hu, J, Yamashita Y, Maher ER, Guay-Woodford L, Neumann HPH, Obermuller H, Koenekoop RK, Bergmann C, Bei X, Lewis RA, Katsanis N, Lopes V, Williams DS, Lyons RH, Dang CV, Brito DA, Dias MB, Zhang X, Nurnberg G, Nurnberg P, Pierce E, Jackson P, Antignac C, Saunier S, Roepman R, Dollfus H, Khanna H, and Hildebrandt F. Candidate exome capture identifies mutation of SDCCAG8 as the cause of a retinal-renal ciliopathy. **Nature Genetics**, 2010. 42(10), 840-850 PMID: 20835237 PMCID: PMC2947620.

b. Rademakers R, Baker M, Nicholson AM, Rutherford NJ, Finch N, Soto-Ortolaza A, Lash J, Wider C, Wojtas A, DeJesus-Hernandez M, Adamson J, Kouri N, Sundal C, Shuster EA, Aasly J, MacKenzie J, Roeber S, Kretzschmar HA, Boeve BF, Knopman DS, Petersen RC, Cairns NJ, Ghetti B, Spina S, Garbern J, Tselis AC, Uitti R, Das P, Van Gerpen JA, Meschia JF, Levy S, Broderick DF, Graff-Radford N, Ross OA, Miller BB, Swerdlow RH, Dickson DW, Wszolek ZK.Mutations in the colony stimulating factor 1 receptor (CSF1R) cause hereditary diffuse leukoencephalopathy with spheroids. **Nature Genetics**. 2011. 44(2), 200-205. PMID: 22197934 PMCID: PMC3267847.

c. Fiskerstrand T, Arshad N, Haukanes BI, Tronstad RR, Pham KDC, Johansson S, Håvik B, Tønder SL, Levy SE, Brackman D, Boman H, Biswas KH, Apold J, Hovdenak N, Visweswariah SS, and Knappskog PM. Familial Diarrhea Syndrome Caused by an Activating GUCY2C Mutation. **New England Journal of Medicine**. 2012. 366(17), 1586-1595. PMID: 22436048.

d. Carlson J, Scott LJ, Locke AE, Flickinger M, Levy S, Myers RM, Boehnke M, Kang HM, Li JZ, Zöllner S. Extremely rare variants reveal patterns of germline mutation rate heterogeneity in humans. **bioRxiv**. 2017:108290.

e. Chao HT, Davids M, Burke E, Pappas JG, Rosenfeld JA, McCarty AJ, Davis T, Wolfe L, Toro C, Tifft C, Xia F, Stong N, Johnson TK, Warr CG, Undiagnosed Diseases N, Yamamoto S, Adams DR, Markello TC, Gahl WA, Bellen HJ, Wangler MF, Malicdan MC. A Syndromic Neurodevelopmental Disorder Caused by De Novo Variants in EBF3. **Am J Hum Genet**. 2017;100(1):128-37. doi: 10.1016/j.ajhg.2016.11.018. PubMed PMID: 28017372; PMCID: PMC5223093.

Education

College
University of New Hampshire: BS, 1994   (Biochemistry, Microbiology)
GPA 3.37
Honors Graduate, Dean's list.

Graduate School
Emory University: PhD, 2000, (Biochemistry)
GPA 3.75
Thesis title: "Genetic Alteration of the Mouse Mitochondrial Genome and Effects on Gene Expression."
Thesis advisor: Professor Douglas C. Wallace

Post-Graduate Training
Emory University, Douglas C. Wallace, March 2000-July 2000

Academic Appointments

Research Assistant Professor, Department of Molecular Physiology and Biophysics,
Vanderbilt University Medical Center, Nashville, TN, July 2000-June 2003

Adjunct Faculty, Graduate training program, Department of Biomedical Informatics, Vanderbilt University Medical Center, Nashville, TN, January 2001-June 2003

Director, Vanderbilt Microarray Shared Resource, Vanderbilt University Medical Center, Nashville, TN, July 2000-August 2009

Assistant Professor, Department of Biomedical Informatics, Vanderbilt University Medical Center, Nashville, TN, July 2003-August 2009. *(Primary Appointment)*

Assistant Professor, Department of Molecular Physiology and Biophysics, Vanderbilt University Medical Center, Nashville, TN, July 2003-August 2009 *(Secondary Appointment)*

Adjunct Associate Professor, Department of Biomedical Informatics, Vanderbilt University Medical Center, Nashville, TN, August 2009-Present

Adjunct Associate Professor, Department of Epidemiology, University of Alabama-Birmingham, Birmingham, AL October 2010-Present.

Adjunct Assistant Professor, Department of Genetics, University of Alabama-Birmingham, Birmingham, AL October 2010-Present.
Adjunct Associate Professor, Department of Biological Sciences, University of Alabama-Huntsville, Huntsville, AL January 2014-Present.

Faculty Investigator, HudsonAlpha Institute for Biotechnology, Huntsville, AL, August 2009-Present

Executive Director, HudsonAlpha Clinical Services Laboratory, LLC, Huntsville, AL, December 2014-Present

Professional Organizations

American Medical Informatics Association, Co-chair, Genomics Working Group (2006-2007)
Association of Biomedical Resource Facilities
American Association for the Advancement of Science
American Association for Cancer Research
American Society for Human Genetics

Professional Activities

Intramural-University
Vision 2020 Personalized Medicine Committee-Task Force 3 (2009)

Intramural-Departmental
Department of Biomedical Informatics Academic Progress Committee (2005-2007)
Department of Biomedical Informatics Curriculum Committee (2007-2009)

Intramural-Center Affiliations
Vanderbilt-Ingram Cancer Center, Associate Member (2000-2009)
Vanderbilt Diabetes Research and Training Center, Member (2000-2009)

Vanderbilt Digestive Disease Research Center, Member (2003-2009)
Vanderbilt Institute of Chemical Biology, Member (2004-2009)

Extramural-Journal Review
▪Reviewer- Arteriosclerosis, Thrombosis and Vascular Biology (2001-present)
▪Reviewer-Bioinformatics (2001-present)
▪Reviewer-Journal of Biological Chemistry (2002-present)
▪Reviewer-Neuropsychopharmacology (2003-present)
▪Reviewer-Kidney International (2003-present)
▪Reviewer-Circulation Research (2003-present)
▪Reviewer-Proceedings of the National Academy of Sciences (2004-present)
▪Reviewer-Mitochondrion (2004-present)
▪Reviewer-Molecular Nutrition and Food Research (2005-present)
▪Reviewer-Pattern Recognition Letters (2006-present)
▪Reviewer-PLOS-Genetics (2006-present)
▪Reviewer-Physiological Genomics (2008-present)
▪Reviewer-Genome Biology (2008-present)

Extramural-Editorial
▪ Member, Editorial Board- Journal of the American Informatics Association (2005-2007)

Extramural-Grant Study Section
▪Reviewer- Alzheimer's Association (2002-present).
▪NIDDK study section ZDK1 GRB-6 "Digestive Disease Research Development Centers" December 2002.
▪NIDDK study section ZDK1 GRB-6 "Digestive Disease Research Development Centers" April 2004.
▪NCI study section ZCA1 SRRB-C "Innovative Technologies for the Detection of Cancer" July 2004.
▪NLM special study section-P41 Biomedical Informatics Resource Grants, April 2005.
▪NLM special emphasis panel ZLM1 HS RO1, July 2005
▪NIH CSR shared equipment study section ZRG1 GGG-T (30, 31), November 2005.
▪DOD Ovarian Cancer Review Panel OC-2, August 2006
▪NIH Special Emphasis Panel ZRG1 GGG-T Genomics and Genetics Shared Instrumentation, October 2006.
▪NCI study section ZCA1 SRRB-U Development of Advanced Genomic Characterization Technologies, November 2006.
▪NIDDK DK-06-017 "Silvio O. Conte Digestive Diseases Research Core Centers P30", June 2007.
▪NIH Special Emphasis Panel ZRG1 GGG-A (30) - S10s genomics and proteomics shared instrumentation, July 2007.
▪NIH Special Emphasis Panel ZRG1 GGG-B (30) - S10s genomics and proteomics shared instrumentation, September 2008.
▪NIAAA Special Review Panel ZAA1-GG-01, November 2008
▪NIH Special Emphasis Panel ZRG1 GGG-A (30) – Genes Genomes and Genetics instrumentation, October 2010.
▪NIH Study Section 2011/05 GHD-Genetics of Health and Disease Study Section, February 2011.
▪NIGRI Study Section 2012/05 ZHG1 HGR-P (M1) 1-H3 AFRICA Initiative, March 2012.

Extramural-Other Review

▪Reviewer, American Association for the Advancement of Science Research Competitive Service-*Microarray Facilities for the Vermont Genetics Network*. April 2002.
▪Reviewer, American Association for the Advancement of Science Research Competitive Service-*External Review of the Michigan Core Technology Alliance*. April 2003.
▪Reviewer, American Association for the Advancement of Science Research Competitive Service-*External Review of the Michigan Core Technology Alliance*. April 2004.
▪Reviewer, American Association for the Advancement of Science Research Competitive Service-*External Review of the Rhode Island EPScOR*. January 2007.
▪Reviewer, American Association for the Advancement of Science Research Competitive Service-*External Review of the Rhode Island EPScOR*. March 2008.
▪Reviewer, American Association for the Advancement of Science Research Competitive Service- *Review of Washington State Life Sciences Discovery Fund* June 2008.
▪Reviewer, American Association for the Advancement of Science Research Competitive Service- *Review of Missouri Life Sciences Research Board* October 2008
▪Reviewer, American Association for the Advancement of Science Research Competitive Service-*External Review of the Rhode Island EPScOR*. June 2009.
▪Reviewer, American Association for the Advancement of Science Research Competitive Service-*External Review of the Rhode Island EPScOR*. May 2010.
▪Reviewer, American Association for the Advancement of Science Research Competitive Service-*External Review of the Rhode Island EPScOR*. September 2011.

Extramural-Advisory
▪Member, Scientific Advisory Board, NuGen Technologies, Inc, San Carlos, CA, October 2003-December 2010.
▪Member, Scientific Advisory Board, Genome Quebec Innovation Centre, Montreal, Quebec, 2008-2011.
▪Member, Scientific Advisory Board, Genomic Explorations Inc, Memphis, TN, 2006-present.
▪Member, Scientific Advisory Board, Rubicon Genomics, Ann Arbor, MI 2013-present.
▪Chairman, Scientific Advisory Board, RainDance Technologies (BioRad), Billerica, MA 2015-present.

Honors and Awards
▪Scholar Athlete, University of New Hampshire, 1993-1994.
▪Dean's list, University of New Hampshire, 1992-1994.
▪Career Development Award, SPORE in Gastrointestinal Cancer 2004-2005
▪Co-Chair, Genomics Working Group of the American Medical Informatics Association 2006-2007.

Teaching Activities

Graduate School Courses as Course Director
BMIF 310-Foundations of Bioinformatics and Computational Biology, 28 lectures, Spring 2004
BMIF 311-Introduction to Systems Biology, 28 lectures, Spring 2009. *This course was a newly developed course for 2009.*

Graduate School Courses as Lecturer
MPB 322-Regulation of Gene Expression, 3 lectures, Spring 2002
MPB 322-Regulation of Gene Expression, 2 lectures, Spring 2003
MPB 322-Regulation of Gene Expression, 3 lectures, Spring 2004

IGP 301-Methodology, 1 lecture, Fall 2004
IGP 301-Methodology, 1 lecture, Fall 2005
IGP 301-Methodology, 1 lecture, Fall 2006
MIM 351-Functional Genomics and Proteomics, 2 lectures, Spring 2006
BMIF 310-Foundations of Bioinformatics and Computational Biology, 7 lectures, Fall 2007
BMIF 310-Foundations of Bioinformatics and Computational Biology, 7 lectures, Fall 2008
BMIF 310-Foundations of Bioinformatics and Computational Biology, 4 lectures, Fall 2009
BMIF 310-Foundations of Bioinformatics and Computational Biology, 4 lectures, Fall 2010
BMIF 310-Foundations of Bioinformatics and Computational Biology, 1 lecture, Fall 2011

Research Supervision

Ph.D. Thesis Committee Member
Stephen VonStetina-Vanderbilt University (2001-2005)
Laura Wilding-Vanderbilt University (2003-2007)
Alex Statnikov-Vanderbilt University (2005-2008)
Alisha Russell-Vanderbilt University (2006-2010)
Mawuli Nyaku-University of Alabama-Birmingham (2010-2014)

M.S. Thesis Committee Member
Alex Statnikov (2003-2005)
Joel Parker (2000-2002)

Student Mentorship
Shristi Shrestha, PhD student (2014-present)
Nripesh Prasad, PhD student (2010-2014)
Sidd Pratrap MS student (2005-2007)
Current position: Director of Bioinformatics, Meharry Medical College, Nashville, TN

Fellow Mentorship
Lewis Frey, PhD (2004-2006)
Current position: Assistant Professor, Department of Biomedical Informatics, University of Utah, Salt Lake City, UT.

Patents Awarded

Multiplex spatial profiling of gene expression
US 7,569,392 B2

Research Support

<u>**ACTIVE**</u>

NIH RFA-HG-16-011 (Cooper/Barsh/Korf)   06/01/2017 – 05/31/2021   0.60 calendar months
$2,840,944
Clinical sequencing across communities in the Deep South

This proposal outlines an important study to apply WGS to diagnose neonates with rare disorders, increase participation of individuals from underrepresented racial/ethnic groups in genomics clinical trials, provide educational materials appropriate to diverse audiences, equip non-genetics healthcare providers to return WGS results, assess the impact of WGS testing and

results, and engage a broad community to implement safer, more effective, and more equitably distributed genomic medicine.

1U24HD090744-01 (Levy/Zhang)	09/23/2016 – 06/30/2019	2.40 calendar months
NIH/NICHD	$6,212,400	

Characterizing pediatric genomes through an optimized sequencing approach

Understanding the fundamental genetic changes associated with structural birth defects and childhood cancers is an important step in developing tools to allow more advanced prediction, treatment and prevention of these devastating conditions. We propose to combine the resources of two world-class centers to support researchers in their investigations of the genetics of birth defects and childhood cancers. This centralized resource will provide researchers with the tools and support necessary to advance our understanding and drive us closer to curing or preventing these diseases.

5UL1TR001417-02 (Kimberly)	08/18/2015 - 03/31/2019	0.60 calendar months
NIH/NCATS	$83,644	

UAB Center for Clinical and Translational Science (CCTS)

The UAB CCTS will enhance human health by driving scientific discovery and dialogue across the bench, bedside and community continuum. The CCTS support this overall mission in a highly integrative network of relationships. Success in creating such an environment is dependent upon success in achieving five strategic priorities: 1) enhancing research infrastructure; 2) promoting investigator education, training and development; 3) accelerating discovery across the T1 interface; 4) expanding value-added partnerships; and 5) building sustainability.

HHSN2722012000231 (Creech)	09/01/2015 – 09/30/2018	0.24 calendar months
NIH/NIAID	$555,660	

Influenza A/H7N9 Vaccine Administered with/without AS03 Adjuvant: Standard and Systems Biology

HudsonAlpha will receive human RNA samples from Vanderbilt University Medical Center. RNA-sequencing will be performed per specifications provided in the clinical protocol and clarified in the manual of procedures. We will perform all necessary experiments, including quality control assays. Once sequencing data are obtained, these FastQ/BAM files will be transferred to Vanderbilt University Medical Center and to the DMID Statistics and Data Coordinating Center (SDCC) for data analysis.

HHSN2722012000231 (Creech)	09/01/2015 – 09/30/2018	0.24 calendar months
NIH/NIAID	$56,630	

Sub-study for DMID 10-0074

HudsonAlpha will receive human RNA samples from Vanderbilt University Medical Center. RNA-sequencing will be performed per specifications provided in the clinical protocol and clarified in the manual of procedures. We will perform all necessary experiments, including quality control assays. Once sequencing data are obtained, these FastQ/BAM files will be transferred to Vanderbilt University Medical Center and to the DMID Statistics and Data Coordinating Center (SDCC) for data analysis.

6U19CA179514-05 (Coffey)	09/01/2013 - 08/31/2018	0.24 calendar months
NIH/NCI	$39,254	

Secreted RNA during CRC progression biogenesis function and clinical markers

Dr. Levy's laboratory will fully support RNA sequencing on 48-74 samples per year prepared from either total RNA or microRNA at the HudsonAlpha Institute for Biotechnology. Dr. Levy's laboratory will provide all required reagents, personnel and basic analysis support for the

proposed sequencing studies during years 1-5 of the project period.

5U01MH105653-03 (Boehnke)   09/19/2014 - 05/31/2018   0.60 calendar months
NIH/NIMH $23,557

Whole Genome Sequencing for Schizophrenia and Bipolar Disorder in the GPC

Dr. Levy will participate in weekly conference calls and several yearly face-to-face meetings to help make this project successful. Any new improvements in sequencing technology, data analysis and data interpretation that are developed and/or applied at HudsonAlpha will be made immediately available to this project.

3P30CA013145-44S4 (Partridge)   04/01/2017 – 03/31/2018   0.60 calendar months
NIH/NCI $113,863

Comprehensive Cancer Center Core Support Grant

Dr. Myers, President and Science Director of HudsonAlpha Institute for Biotechnology, will be part of the director's council. The director's council meets on a monthly basis to advise the director on all major decisions regarding the UAB-CCC, its organization, planning and evaluation and to approve new developmental research programs and review program leaderships. In addition, Dr. Myers will co-lead UAB-CCC's Experimental Therapeutics program. Drs. Absher and Levy will be co-leaders of the Cancer Cell Biology Program and Cancer Control & Population Sciences Program. Dr. Cooper is an Associate Scientist in Experimental Therapeutics program. They will consult investigators in study design and analysis related to genomic data.

4UM1HG007301-04 (Cooper/Myers)   06/14/2013-05/31/2018(NCE)0.60 calendar months
NIH/NHGRI $1,536,927

Genomic Diagnosis in Children with Developmental Delay

The goal of this project is to address technological, analytical, and ethical challenges that prevent optimal use of DNA sequencing to improve treatment of diseases and life planning for patients and their families. We are applying next-generation DNA sequencing to meet the diagnostic needs of children with developmental delay, intellectual disability and related health problems.

Genomic Services Lab Director   4.80 calendar months

In addition to the projects listed above, Dr. Levy, as the Director of the Genomic Services Laboratory (GSL), is involved in the development and application of genomic and bioinformatic technologies and methods to support scientific research. These activities, along with fee-for-service projects, change often making it difficult to assign a precise percent effort to individual projects. Dr. Levy has reviewed his GSL obligations and confirms that the aggregate effort on all GSL projects at any given time does not exceed 40% (4.80 calendar months) of institutional effort.

PENDING

COMPLETED

US MED Research ACQ Activity (PI: Richard M. Myers)
9/16/10 - 8/31/15
Direct Costs for current year: $2,150,777
Shawn E. Levy effort: 33% effort [4.0 cal. mos.]
Title: Global genomic analysis of prostate, breast and pancreatic cancer

The goals of this study are to provide an unprecedented comprehensive view of the molecular pathogenesis of prostate, breast, and pancreatic cancer, as well as the differential response to treatments in breast cancer. We will use next-generation DNA sequencing to measure mRNA, microRNA, DNA methylation, DNase hypersensitivity sites, histone modifications, and sites of transcription factor occupancy in tumors and matched non-tumor tissues for these three cancers. No budgetary or scientific overlap.
Role: Co-investigator

NIH (PIs of Collaborative R01: Richard M. Myers and Michael Boehnke)
8/30/11 - 6/30/14
Direct costs for current year for HudsonAlpha portion: $1,855,348
Shawn E. Levy effort: 20% [2.4 cal. mos.]
Title: Whole Genome and Exome Sequencing for Bipolar Disorder
In this collaborative R01 grant, performed jointly with Dr. Michael Boehnke and colleagues at the University of Michigan, we are performing a detailed genetic analysis of bipolar disorder. We are using ultrahigh-throughput sequencing to determine the deep whole genome sequences from 1,000 individuals with bipolar disorder and 1,000 control individuals without the disorder.

NIH/NIAMS 1 R01 AR057202 (PI: Louis Bridges)
4/1/09 - 3/31/14
Direct Costs for current year for Myers/Absher portion: $298,704
Shawn E. Levy effort: 5% effort [0.60 cal mos.]
Title: Genome Wide Association Study in African-Americans with Rheumatoid Arthritis
In this study, the Myers lab and Devin Absher and his lab at HudsonAlpha are collaborating with Dr. Lou Bridges and his colleagues at the School of Medicine at the University of Alabama in Birmingham to perform a genome-wide genetic association study of rheumatoid arthritis in African Americans. No budgetary or scientific overlap.
Role: Co-investigator

NHGRI P50 HG02568 (PI: David Kingsley)
4/19/02 - 5/31/12
Direct costs for current year: $701,981
Shawn E. Levy effort: 10% effort [1.2 cal. mos.]
Title: Center for Vertebrate Diversity
The continuation of this Center of Excellence in Genome Science (CEGS) has broad goals to understand the genetic basis for the striking biological diversity seen in vertebrate animals. We use genetics, genomics, molecular biology and computational tools to study this problem, focusing on the three-spined stickleback fish.   HudsonAlpha performs many of the genomic experiments for this project, including genomic DNA sequencing, cDNA sequencing, BAC map construction, and genotyping.
Role: Co-investigator

5 U54 HG004576-03 (Myers)   10/01/2007 – 09/30/2011   1.20 calendar
NIH/NHGRI $3,985,643
"Global Annotation of Regulatory Elements in the Human Genome"

This project, which is a collaboration between the Myers group at HudsonAlpha and Barbara Wold's group at Caltech, along with contributions from Wing Wong, Arend Sidow, Serafim Batzoglou and Gavin Sherlock at Stanford, is part of the ENCODE Project, whose goals are to identify and understand the roles of all the functional elements throughout the entire human

genome. Our contributions are to identify transcription factor binding sites, assess the methylation status and measure RNAs with next-gen sequencing.
Role: Co-investigator

1 RC1 DK086594-01 (Southard-Smith)
09/30/2009 – 09/29/2011   0.60 calendar months
NIH $240,970
"Gene Networks in Neutral Crest-derived Innervation   of the Lower Urinary Tract"
The studies proposed aim to identify essential genes that control development of nerves in the lower urinary tract that regulate bladder control and sexual function. These studies are important for understanding how these nerves normally develop and for deriving technologies that will restore neural function in urogenital birth defects or after pelvic surgery. This proposal is in response to the broad Challenge grant area of Regenerative medicine and meets multiple needs for basic research in development lower urinary tract innervation.
Role: Co-investigator

5 P30 CA68485-13 (Pietenpol)

09/28/2004 - 08/31/2009   1.80 calendar months
NIH/NCI $3,553,801
"Cancer Center Support Grant"

As part of the Vanderbilt Ingram Cancer Center's support grant, the goal of the Microarray Core is to provide genome-scale expression profiling technologies as well as analysis and informatics support to researchers who are members of the center.

5 P30DK058404-07 (Polk)
08/30/2007 - 05/31/2012   1.20 calendar months
NIH/NIDDK $727,500
"Molecular and Cellular Basis of Digestive Diseases"

As part of a center grant, the goal of the Microarray Core in the Vanderbilt Digestive Diseases Center is to provide support for the use of genome-scale expression profiling technologies to researchers involved in digestive disease-related research.
Role: Core Leader

5 P60 DK20593-31 (Powers)
06/01/2007 - 03/31/2012   0.24 calendar months
NIH/NIDDK $1,487,659
"Diabetes Research and Training Center"

As part of a center grant, the goal of the Microarray and Bioinformatics Core in the Diabetes Research and Training Center is to provide support for the use of genome-scale expression profiling technologies to researchers involved in DRTC-related research.
Role: Core Leader

2 R01 CA064277-10A1 (Zheng)
08/05/2008 - 05/31/2013   0.24 calendar months
NIH/NCI $324,917
"Shanghai Breast Cancer Study"

This proposal is aimed at the development of novel algorithms for the analysis of high-dimensionality data towards to the discovery of causal markers and mechanisms.
Role: Co-investigator

5 U24 DK58749-03   (George)

09/30/00 - 08/31/03   1.2 calendar months
NIH/NIDDK
Vanderbilt NIDDK Biotechnology Center
Purpose: The goal of this proposal was the establishment of a Biotechnology Center for the support of genomic studies of interest to investigators funded by the NIDDK. Microarray technologies and related informatics were central to the efforts.
Role: Co-investigator

VUMC Discovery Grant 540   (Levy)

01/01/02 - 12/31/03   1.2 calendar months
VUMC Internal Grant $50,000
Gene Expression Analysis of Colon Cancer
The goal of this proposal was the development of an integrated RNA and protein expression profile for colon cancer utilizing microarray and high-resolution protein profiling technologies. These profiles were useful in designing and developing both technological and informatic platforms for the combined analysis of protein and genetic profiles of cancer.
Role: Principle Investigator

ACS IRG-58-009-46   (Levy)   07/01/03 - 06/30/04

ACS/VICC
Simultaneous profiling of protein and RNA expression by mass spectrometry in intact tissue sections.
The goal of this proposal is to develop a novel technology platform that facilitates the simultaneous profiling of protein and RNA species in intact tissue samples while reporting spatial position. This will provide an unprecedented resolution to examine the biology of tumor samples and host-tumor interactions.
Role: Principle Investigator

1 R21 NS043581-01A1   (McDonald)

12/01/02 - 11/30/04
NIH/NINDS
Gene Discovery in a Putative Mouse Model of ADHD
In this proposal, microarray technology will be used to examine differential gene expression in the mouse model of ADHD, providing a rare opportunity to discover genes downstream of TRß activity that are able to produce all of the core symptoms and many adjunct features of ADHD.
Role: Co-investigator

1 U01 DK063587-01   (Hayward)

09/30/02 - 06/30/05
NIH/NIDDK
Genetic Markers of Transition Zone Hyperplasia
The goals of this proposal are the identification of biomarkers for prostate hyperplasia through the use of high-density microarray studies on novel models of prostate disease.
Role: Co-investigator

W81XWH-04-1-0626 (Levy S)
07/15/04-07/14/06
Department of Defense
Simultaneous profiling of protein and RNA expression by mass spectrometry in intact breast tissue sections.
The goal of this proposal is to continue the development of a novel technology platform that facilitates the simultaneous profiling of protein and RNA species in intact tissue samples while reporting spatial position. This proposal will specifically fund the optimization of this technology for the analysis of breast tissue samples.
Role: Principle Investigator

5 P01 HL6744-04 (Hawiger J)
12/01/01-11/30/06
NIH/NHLBI
Functional Genomics of Inflammation
As part of a Program Project Grant, the goal of the Microarray Core in the Functional Genomics of Inflammation program project is to provide genome-scale expression profiling technologies to researchers involved in the program.
Role: Core Leader

1 R01 DK068261-01 (Nagy T)
07/01/04-06/30/07
NIH/NIDDK (subcontract with UT)
Antipsychotic Drug-induced Weight Gain
The goal of this study is to understand the actions of antipsychotic drugs as they alter body weight. In this short subcontract with the University of Alabama, an animal model system used to study the molecular effects of selected drugs will be analyzed using genomic profiling techniques.
Role: Principal Investigator-subcontract

5 P60 DK20593-27 (Powers A)
07/20/02-03/31/07
NIH/NIDDK
Diabetes Research and Training Center-Microarray and Bioinformatics Core
As part of a center grant, the goal of the Microarray Core in the Diabetes Research and Training Center is to provide support for the use of genome-scale expression profiling technologies to researchers involved in DRTC-related research.
Role: Core Leader

5 P50 CA95103-04 (Coffey RJ)
09/24/02-04/30/07
NIH/NCI
SPORE in GI Cancer
This study will investigate the molecular features of tumors in GI cancer and provide full support for genomic profiling projects as part of the overall SPORE program.
Role: Core Leader

U24 CA126563 (Myers)
09/28/06 – 08/31/10
NIH/NCI
"The HudsonAlpha Cancer Genome Characterization Center

We are characterizing tumors and matched non-tumor samples for copy number variations throughout the human genome as part of The Cancer Genome Atlas project, a trans-NIH initiative aimed at learning all the genetic and genomic changes associated with cancer. We use a whole-genome genotyping method to assay more than 1 million SNPs throughout the genome.
Role: Co-investigator

1 RC1 HL100016-01 (Schey)
09/30/09 – 09/29/11
NIH-ARRA Funding
"Proteome and Transcriptome Markers of Hypertension in Urine and Plasma Exosomes"
The goal of the proposed research is to develop a novel method for discovery of molecular markers of disease that circumvents existing obstacles. Through analysis of proteins and RNA found in lipid particles isolated from blood and urine, new markers of disease will be discovered that improve diagnosis, prognosis, and prediction of response to therapy; that is, improve personalized medicine. The new methodology will be applied to reveal biomarkers of salt-sensitivity and therapeutic response in hypertensive subjects.
Role: Co-investigator

Publications

162 peer-reviewed publications with a total of 23,891 citations (*as of October 2018*)**.**

> *A full publication and patent listing can be accessed via a public Google Scholar profile at:*
> *http://scholar.google.com/citations?user=xeKJAZ0AAAAJ*
> *As well as at NCBI:*
> *http://www.ncbi.nlm.nih.gov/sites/myncbi/1BODvQqGn4iAa/bibliography/43127950/public/*

Articles in refereed journals

1. Levy SE, Waymire KG, Kim YL, MacGregor GR, Wallace DC. Transfer of chloramphenicol-resistant mitochondrial DNA into the chimeric mouse. **Transgenic Res**. 1999;8(2):137-145. PubMed PMID: 10481313; PMCID: PMC3049807.

2. Levy SE, Chen YS, Graham BH, Wallace DC. Expression and sequence analysis of the mouse adenine nucleotide translocase 1 and 2 genes. **Gene**. 2000;254(1-2):57-66. PubMed PMID: 10974536.

3. Sligh JE, Levy SE, Waymire KG, Allard P, Dillehay DL, Nusinowitz S, Heckenlively JR, MacGregor GR, Wallace DC. Maternal germ-line transmission of mutant mtDNAs from embryonic stem cell-derived chimeric mice. **Proc Natl Acad Sci U S A**. 2000;97(26):14461-14466. doi: 10.1073/pnas.250491597. PubMed PMID: 11106380; PMCID: PMC18941.

4. Levy SE, Muldowney JA, 3rd. Microarray analysis of neointima: flowing toward a clear future. **Arterioscler Thromb Vasc Biol**. 2002;22(12):1946-1947. PubMed PMID: 12482816.

5. Park Y-K, Franklin JL, Settle SH, Levy SE, Whitehead RH, Coffey RJ. Microarray gene expression profiling and correlation with morphological changes during mouse colonic development. **Gastroenterology**. 2003;124(4):A602.

6. Gu G, Deutch AY, Franklin J, Levy S, Wallace DC, Zhang J. Profiling genes related to mitochondrial function in mice treated with N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. **Biochem Biophys Res Commun**. 2003;308(1):197-205. PubMed PMID: 12890501.

7. Yamagata N, Shyr Y, Yanagisawa K, Edgerton M, Dang TP, Gonzalez A, Nadaf S, Larsen P, Roberts JR, Nesbitt JC, Jensen R, Levy S, Moore JH, Minna JD, Carbone DP. A training-testing approach to the molecular classification of resected non-small cell lung cancer. **Clin Cancer Res**. 2003;9(13):4695-4704. PubMed PMID: 14581339.

8. Levy SE. Microarray analysis in drug discovery: an uplifting view of depression. **Sci STKE**. 2003;2003(206):pe46. doi: 10.1126/stke.2003.206.pe46. PubMed PMID: 14583588.

9. McQuain MK, Seale K, Peek J, Levy S, Haselton FR. Effects of relative humidity and buffer additives on the contact printing of microarrays by quill pins. **Anal Biochem**. 2003;320(2):281-291. PubMed PMID: 12927835.

10. McQuain MK, Seale K, Peek J, Fisher TS, Levy S, Stremler MA, Haselton FR. Chaotic mixer improves microarray hybridization. **Anal Biochem**. 2004;325(2):215-226. PubMed PMID: 14751256.

11. Law AK, Gupta D, Levy S, Wallace DC, McKeon RJ, Buck CR. TGF-beta1 induction of the adenine nucleotide translocator 1 in astrocytes occurs through Smads and Sp1 transcription factors. **BMC Neurosci**. 2004;5:1. doi: 10.1186/1471-2202-5-1. PubMed PMID: 14720305; PMCID: PMC324399.

12. Kokoszka JE, Waymire KG, Levy SE, Sligh JE, Cai J, Jones DP, MacGregor GR, Wallace DC. The ADP/ATP translocator is not essential for the mitochondrial permeability transition pore. **Nature**. 2004;427(6973):461-465. doi: 10.1038/nature02229. PubMed PMID: 14749836; PMCID: PMC3049806.

13. Sforza DM, Annese J, Liu D, Levy S, Toga AW, Smith DJ. Anatomical methods for voxelation of the mammalian brain. **Neurochem Res**. 2004;29(6):1299-1306. PubMed PMID: 15176486.

14. Friedman DB, Hill S, Keller JW, Merchant NB, Levy SE, Coffey RJ, Caprioli RM. Proteome analysis of human colon cancer by two-dimensional difference gel electrophoresis and mass spectrometry. **Proteomics**. 2004;4(3):793-811. doi: 10.1002/pmic.200300635. PubMed PMID: 14997500.

15. Chung C, Carter J, Ely K, Murphy B, Burkey B, Netterville J, Levy S, Cmelak A, Slebos R, Yarbrough W. Gene expression analysis of head and neck squamous cell carcinoma using formalin-fixed paraffin embedded tissue. **Journal of Clinical Oncology**. 2005;23(16_suppl):5537-5537.

16. Levy SE, Statnikov A, Aliferis C. Biomarker selection from high-dimensionality data. **Pharmaceutical Discovery**. 2005;5(7):S37-S37.

17. Park YK, Franklin JL, Settle SH, Levy SE, Chung E, Jeyakumar LH, Shyr Y, Washington MK, Whitehead RH, Aronow BJ, Coffey RJ. Gene expression profile analysis of mouse colon embryonic development. **Genesis**. 2005;41(1):1-12. doi: 10.1002/gene.20088. PubMed PMID: 15645444.

18. Statnikov A, Aliferis CF, Tsamardinos I, Hardin D, Levy S. A comprehensive evaluation of multicategory classification methods for microarray gene expression cancer diagnosis. **Bioinformatics**. 2005;21(5):631-643. doi: 10.1093/bioinformatics/bti033. PubMed PMID: 15374862.

19. Xie L, Xu BJ, Gorska AE, Shyr Y, Schwartz SA, Cheng N, Levy S, Bierie B, Caprioli RM, Moses HL. Genomic and proteomic analysis of mammary tumors arising in transgenic mice. **J Proteome Res**. 2005;4(6):2088-2098. doi: 10.1021/pr050214l. PubMed PMID: 16335954.

20. Chung CH, Levy S, Yarbrough WG. Clinical applications of genomics in head and neck cancer. **Head Neck**. 2006;28(4):360-368. doi: 10.1002/hed.20323. PubMed PMID: 16284976.

21. Acuff HB, Sinnamon M, Fingleton B, Boone B, Levy SE, Chen X, Pozzi A, Carbone DP, Schwartz DR, Moin K, Sloane BF, Matrisian LM. Analysis of host- and tumor-derived proteinases using a custom dual species microarray reveals a protective role for stromal matrix metalloproteinase-12 in non-small cell lung cancer. **Cancer Res**. 2006;66(16):7968-7975. doi: 10.1158/0008-5472.CAN-05-4279. PubMed PMID: 16912171.

22. Chung CH, Parker JS, Ely K, Carter J, Yi Y, Murphy BA, Ang KK, El-Naggar AK, Zanation AM, Cmelak AJ, Levy S, Slebos RJ, Yarbrough WG. Gene expression profiles identify epithelial-to-mesenchymal transition and activation of nuclear factor-kappaB signaling as characteristics of a high-risk head and neck squamous cell carcinoma. **Cancer Res**. 2006;66(16):8210-8218. doi: 10.1158/0008-5472.CAN-06-1213. PubMed PMID: 16912200.

23. Slebos RJ, Yi Y, Ely K, Carter J, Evjen A, Zhang X, Shyr Y, Murphy BM, Cmelak AJ, Burkey BB, Netterville JL, Levy S, Yarbrough WG, Chung CH. Gene expression differences associated with human papillomavirus status in head and neck squamous cell carcinoma. **Clin Cancer Res**. 2006;12(3 Pt 1):701-709. doi: 10.1158/1078-0432.CCR-05-2017. PubMed PMID: 16467079.

24. Consortium M, Shi L, Reid LH, Jones WD, Shippy R, Warrington JA, Baker SC, Collins PJ, de Longueville F, Kawasaki ES, Lee KY, Luo Y, Sun YA, Willey JC, Setterquist RA, Fischer GM, Tong W, Dragan YP, Dix DJ, Frueh FW, Goodsaid FM, Herman D, Jensen RV, Johnson CD, Lobenhofer EK, Puri RK, Schrf U, Thierry-Mieg J, Wang C, Wilson M, Wolber PK, Zhang L, Amur S, Bao W, Barbacioru CC, Lucas AB, Bertholet V, Boysen C, Bromley B, Brown D, Brunner A, Canales R, Cao XM, Cebula TA, Chen JJ, Cheng J, Chu TM, Chudin E, Corson J, Corton JC, Croner LJ, Davies C, Davison TS, Delenstarr G, Deng X, Dorris D, Eklund AC, Fan XH, Fang H, Fulmer-Smentek S, Fuscoe JC, Gallagher K, Ge W, Guo L, Guo X, Hager J, Haje PK, Han J, Han T, Harbottle HC, Harris SC, Hatchwell E, Hauser CA, Hester S, Hong H, Hurban P, Jackson SA, Ji H, Knight CR, Kuo WP, LeClerc JE, Levy S, Li QZ, Liu C, Liu Y, Lombardi MJ, Ma Y, Magnuson SR, Maqsodi B, McDaniel T, Mei N, Myklebost O, Ning B, Novoradovskaya N, Orr MS, Osborn TW, Papallo A, Patterson TA, Perkins RG, Peters EH, Peterson R, Philips KL, Pine PS, Pusztai L, Qian F, Ren H, Rosen M, Rosenzweig BA, Samaha RR, Schena M, Schroth GP, Shchegrova S, Smith DD, Staedtler F, Su Z, Sun H, Szallasi Z, Tezak Z, Thierry-Mieg D, Thompson KL, Tikhonova I, Turpaz Y, Vallanat B, Van C, Walker SJ, Wang SJ, Wang Y, Wolfinger R, Wong A, Wu J, Xiao C, Xie Q, Xu J, Yang W, Zhang L, Zhong S, Zong Y, Slikker W, Jr. The MicroArray Quality Control (MAQC) project shows inter- and intraplatform reproducibility of gene expression measurements. **Nat Biotechnol**. 2006;24(9):1151-1161. doi: 10.1038/nbt1239. PubMed PMID: 16964229; PMCID: PMC3272078.

25. Chung CH, Ely K, McGavran L, Varella-Garcia M, Parker J, Parker N, Jarrett C, Carter J, Murphy BA, Netterville J, Burkey BB, Sinard R, Cmelak A, Levy S, Yarbrough WG, Slebos RJ, Hirsch FR. Increased epidermal growth factor receptor gene copy number is associated with poor prognosis in head and neck squamous cell carcinomas. **J Clin Oncol**. 2006;24(25):4170-4176. doi: 10.1200/JCO.2006.07.2587. PubMed PMID: 16943533.

26. Chung CH, Parker J, Levy S, Slebos RJ, Dicker AP, Rodeck U. Gene expression profiles as markers of aggressive disease-EGFR as a factor. **Int J Radiat Oncol Biol Phys**. 2007;69(2 Suppl):S102-105. doi: 10.1016/j.ijrobp.2007.05.039. PubMed PMID: 17848272; PMCID: PMC2361130.

27. Frey L, Edgerton M, Fisher D, Levy S. Ensemble stump classifiers and gene expression signatures in lung cancer. **Stud Health Technol Inform**. 2007;129(Pt 2):1255-1259. PubMed PMID: 17911916.

28. Kaiser S, Park YK, Franklin JL, Halberg RB, Yu M, Jessen WJ, Freudenberg J, Chen X, Haigis K, Jegga AG, Kong S, Sakthivel B, Xu H, Reichling T, Azhar M, Boivin GP, Roberts RB, Bissahoyo AC, Gonzales F, Bloom GC, Eschrich S, Carter SL, Aronow JE, Kleimeyer J, Kleimeyer M, Ramaswamy V, Settle SH, Boone B, Levy S, Graff JM, Doetschman T, Groden J, Dove WF, Threadgill DW, Yeatman TJ, Coffey RJ, Jr., Aronow BJ. Transcriptional recapitulation and subversion of embryonic colon development by mouse colon tumor models and human colon cancer. **Genome Biol**. 2007;8(7):R131. doi: 10.1186/gb-2007-8-7-r131. PubMed PMID: 17615082; PMCID: PMC2323222.

29. Zhou S, Wei S, Boone B, Levy S. Microarray analysis of genes affected by salt stress in tomato. **African Journal of Environmental Science and Technology**. 2007;1(2):14-26.

30. Chin MH, Geng AB, Khan AH, Qian WJ, Petyuk VA, Boline J, Levy S, Toga AW, Smith RD, Leahy RM, Smith DJ. A genome-scale map of expression for a mouse brain section obtained using voxelation. **Physiol Genomics**. 2007;30(3):313-321. doi: 10.1152/physiolgenomics.00287.2006. PubMed PMID: 17504947; PMCID: PMC3299369.

31. Muldowney JA, 3rd, Stringham JR, Levy SE, Gleaves LA, Eren M, Piana RN, Vaughan DE. Antiproliferative agents alter vascular plasminogen activator inhibitor-1 expression: a potential prothrombotic mechanism of drug-eluting stents. **Arterioscler Thromb Vasc Biol**. 2007;27(2):400-406. doi: 10.1161/01.ATV.0000254677.12861.b8. PubMed PMID: 17158352.

32. Chung CH, Levy S, Chaurand P, Carbone DP. Genomics and proteomics: emerging technologies in clinical cancer research. **Crit Rev Oncol Hematol**. 2007;61(1):1-25. doi: 10.1016/j.critrevonc.2006.06.005. PubMed PMID: 17015021.

33. Joshi S, Davies H, Sims LP, Levy SE, Dean J. Ovarian gene expression in the absence of FIGLA, an oocyte-specific transcription factor. **BMC Dev Biol**. 2007;7:67. doi: 10.1186/1471-213X-7-67. PubMed PMID: 17567914; PMCID: PMC1906760.

34. Zhou S, Sauvé R, Boone B, Levy S. Identification of genes associated with aluminium toxicity in tomato roots using cDNA microarrays. **Plant Stress**. 2008;2(2):113-120.

35. Pratap S, Williams SM, Levy SE. Evaluation of pooled allelotyping versus individual genotyping for genome-wide association analysis of complex disease. **BMC Bioinformatics**. 2008;9(7):P11.

36. Beeghly-Fadiel A, Long JR, Gao YT, Li C, Qu S, Cai Q, Zheng Y, Ruan ZX, Levy SE, Deming SL, Snoddy JR, Shu XO, Lu W, Zheng W. Common MMP-7 polymorphisms and breast cancer susceptibility: a multistage study of association and functionality. **Cancer Res**. 2008;68(15):6453-6459. doi: 10.1158/0008-5472.CAN-08-0636. PubMed PMID: 18648013; PMCID: PMC2718434.

37. Watson JD, Wang S, Von Stetina SE, Spencer WC, Levy S, Dexheimer PJ, Kurn N, Heath JD, Miller DM, 3rd. Complementary RNA amplification methods enhance microarray identification of transcripts expressed in the C. elegans nervous system. **BMC Genomics**.

2008;9:84. doi: 10.1186/1471-2164-9-84. PubMed PMID: 18284693; PMCID: PMC2263045.

38.   Wilding Crawford L, Tweedie Ables E, Oh YA, Boone B, Levy S, Gannon M. Gene expression profiling of a mouse model of pancreatic islet dysmorphogenesis. **PLoS One**. 2008;3(2):e1611. doi: 10.1371/journal.pone.0001611. PubMed PMID: 18297134; PMCID: PMC2249940.

39.   Kanies CL, Smith JJ, Kis C, Schmidt C, Levy S, Khabar KS, Morrow J, Deane N, Dixon DA, Beauchamp RD. Oncogenic Ras and transforming growth factor-beta synergistically regulate AU-rich element-containing mRNAs during epithelial to mesenchymal transition. **Mol Cancer Res**. 2008;6(7):1124-1136. doi: 10.1158/1541-7786.MCR-07-2095. PubMed PMID: 18644977; PMCID: PMC2572152.

40.   Xu B, Roos JL, Levy S, van Rensburg EJ, Gogos JA, Karayiorgou M. Strong association of de novo copy number mutations with sporadic schizophrenia. **Nat Genet**. 2008;40(7):880-885. doi: 10.1038/ng.162. PubMed PMID: 18511947.

41.   Subramaniam V, Golik P, Murdock DG, Levy S, Kerstann KW, Coskun PE, Melkonian GA, Wallace DC. MITOCHIP assessment of differential gene expression in the skeletal muscle of Ant1 knockout mice: coordinate regulation of OXPHOS, antioxidant, and apoptotic genes. **Biochim Biophys Acta**. 2008;1777(7-8):666-675. doi: 10.1016/j.bbabio.2008.03.015. PubMed PMID: 18439414; PMCID: PMC4391341.

42.   Liu YJ, Liu XG, Wang L, Dina C, Yan H, Liu JF, Levy S, Papasian CJ, Drees BM, Hamilton JJ, Meyre D, Delplanque J, Pei YF, Zhang L, Recker RR, Froguel P, Deng HW. Genome-wide association scans identified CTNNBL1 as a novel gene for obesity. **Hum Mol Genet**. 2008;17(12):1803-1813. doi: 10.1093/hmg/ddn072. PubMed PMID: 18325910; PMCID: PMC2900891.

43.   Liu YZ, Wilson SG, Wang L, Liu XG, Guo YF, Li J, Yan H, Deloukas P, Soranzo N, Chinappen-Horsley U, Cervino A, Williams FM, Xiong DH, Zhang YP, Jin TB, Levy S, Papasian CJ, Drees BM, Hamilton JJ, Recker RR, Spector TD, Deng HW. Identification of PLCL1 gene for hip bone size variation in females in a genome-wide association study. **PLoS One**. 2008;3(9):e3160. doi: 10.1371/journal.pone.0003160. PubMed PMID: 18776929; PMCID: PMC2522269.

44.   Hatakeyama H, Parker J, Wheeler D, Harari P, Levy S, Chung C. Effect of insulin-like growth factor 1 receptor inhibitor on sensitization of head and neck cancer cells to cetuximab and methotrexate. **Journal of Clinical Oncology**. 2009;27(15S):6079-6079.

45.   Liu Y-Z, Pei Y-F, Liu J-F, Yang F, Guo Y, Zhang L, Liu X-G, Yan H, Wang L, Zhang Y-P. Powerful bivariate genome-wide association analyses suggest the SOX6 gene influencing both obesity and osteoporosis phenotypes in males. **PloS One**. 2009;4(8):e6827.

46.   Liu YZ, Pei YF, Liu JF, Yang F, Guo Y, Zhang L, Liu XG, Yan H, Wang L, Zhang YP, Levy S, Recker RR, Deng HW. Powerful bivariate genome-wide association analyses suggest the SOX6 gene influencing both obesity and osteoporosis phenotypes in males. **PLoS One**. 2009;4(8):e6827. doi: 10.1371/journal.pone.0006827. PubMed PMID: 19714249; PMCID: PMC2730014.

47.   Liu YZ, Pei YF, Guo YF, Wang L, Liu XG, Yan H, Xiong DH, Zhang YP, Levy S, Li J, Haddock CK, Papasian CJ, Xu Q, Ma JZ, Payne TJ, Recker RR, Li MD, Deng HW. Genome-wide association analyses suggested a novel mechanism for smoking behavior regulated by IL15. **Mol Psychiatry**. 2009;14(7):668-680. doi: 10.1038/mp.2009.3. PubMed PMID: 19188921; PMCID: PMC2700850.

48. Zheng W, Long J, Gao YT, Li C, Zheng Y, Xiang YB, Wen W, Levy S, Deming SL, Haines JL, Gu K, Fair AM, Cai Q, Lu W, Shu XO. Genome-wide association study identifies a new breast cancer susceptibility locus at 6q25.1. **Nat Genet**. 2009;41(3):324-328. doi: 10.1038/ng.318. PubMed PMID: 19219042; PMCID: PMC2754845.

49. Xiong DH, Liu XG, Guo YF, Tan LJ, Wang L, Sha BY, Tang ZH, Pan F, Yang TL, Chen XD, Lei SF, Yerges LM, Zhu XZ, Wheeler VW, Patrick AL, Bunker CH, Guo Y, Yan H, Pei YF, Zhang YP, Levy S, Papasian CJ, Xiao P, Lundberg YW, Recker RR, Liu YZ, Liu YJ, Zmuda JM, Deng HW. Genome-wide association and follow-up replication studies identified ADAMTS18 and TGFBR3 as bone mass candidate genes in different ethnic groups. **Am J Hum Genet**. 2009;84(3):388-398. doi: 10.1016/j.ajhg.2009.01.025. PubMed PMID: 19249006; PMCID: PMC2667986.

50. Liu YZ, Guo YF, Wang L, Tan LJ, Liu XG, Pei YF, Yan H, Xiong DH, Deng FY, Yu N, Zhang YP, Zhang L, Lei SF, Chen XD, Liu HB, Zhu XZ, Levy S, Papasian CJ, Drees BM, Hamilton JJ, Recker RR, Deng HW. Genome-wide association analyses identify SPOCK as a key novel gene underlying age at menarche. **PLoS Genet**. 2009;5(3):e1000420. doi: 10.1371/journal.pgen.1000420. PubMed PMID: 19282985; PMCID: PMC2652107.

51. Liu XG, Tan LJ, Lei SF, Liu YJ, Shen H, Wang L, Yan H, Guo YF, Xiong DH, Chen XD, Pan F, Yang TL, Zhang YP, Guo Y, Tang NL, Zhu XZ, Deng HY, Levy S, Recker RR, Papasian CJ, Deng HW. Genome-wide association and replication studies identified TRHR as an important gene for lean body mass. **Am J Hum Genet**. 2009;84(3):418-423. doi: 10.1016/j.ajhg.2009.02.004. PubMed PMID: 19268274; PMCID: PMC2668008.

52. Lei SF, Tan LJ, Liu XG, Wang L, Yan H, Guo YF, Liu YZ, Xiong DH, Li J, Yang TL, Chen XD, Guo Y, Deng FY, Zhang YP, Zhu XZ, Levy S, Papasian CJ, Hamilton JJ, Recker RR, Deng HW. Genome-wide association study identifies two novel loci containing FLNB and SBF2 genes underlying stature variation. **Hum Mol Genet**. 2009;18(9):1661-1669. doi: 10.1093/hmg/ddn405. PubMed PMID: 19039035; PMCID: PMC2667283.

53. Li J, Yang T, Wang L, Yan H, Zhang Y, Guo Y, Pan F, Zhang Z, Peng Y, Zhou Q, He L, Zhu X, Deng H, Levy S, Papasian CJ, Drees BM, Hamilton JJ, Recker RR, Cheng J, Deng HW. Whole genome distribution and ethnic differentiation of copy number variation in Caucasian and Asian populations. **PLoS One**. 2009;4(11):e7958. doi: 10.1371/journal.pone.0007958. PubMed PMID: 19956714; PMCID: PMC2776354.

54. De Moor MH, Liu YJ, Boomsma DI, Li J, Hamilton JJ, Hottenga JJ, Levy S, Liu XG, Pei YF, Posthuma D, Recker RR, Sullivan PF, Wang L, Willemsen G, Yan H, De Geus EJ, Deng HW. Genome-wide association study of exercise behavior in Dutch and American adults. **Med Sci Sports Exerc**. 2009;41(10):1887-1895. doi: 10.1249/MSS.0b013e3181a2f646. PubMed PMID: 19727025; PMCID: PMC2895958.

55. Canter JA, Robbins GK, Selph D, Clifford DB, Kallianpur AR, Shafer R, Levy S, Murdock DG, Ritchie MD, Haas DW, Hulgan T, Team ACTGS, New Work Concept Sheet T. African mitochondrial DNA subhaplogroups and peripheral neuropathy during antiretroviral therapy. **J Infect Dis**. 2010;201(11):1703-1707. doi: 10.1086/652419. PubMed PMID: 20402593; PMCID: PMC2862090.

56. Greco JA, 3rd, Pollins AC, Boone BE, Levy SE, Nanney LB. A microarray analysis of temporal gene expression profiles in thermally injured human skin. **Burns**. 2010;36(2):192-204. doi: 10.1016/j.burns.2009.06.211. PubMed PMID: 19781859; PMCID: PMC3755600.

57. Smith JJ, Deane NG, Wu F, Merchant NB, Zhang B, Jiang A, Lu P, Johnson JC, Schmidt C, Bailey CE, Eschrich S, Kis C, Levy S, Washington MK, Heslin MJ, Coffey RJ, Yeatman

TJ, Shyr Y, Beauchamp RD. Experimentally derived metastasis gene expression profile predicts recurrence and death in patients with colon cancer. **Gastroenterology**. 2010;138(3):958-968. doi: 10.1053/j.gastro.2009.11.005. PubMed PMID: 19914252; PMCID: PMC3388775.

58. Motsinger-Reif AA, Antas PR, Oki NO, Levy S, Holland SM, Sterling TR. Polymorphisms in IL-1beta, vitamin D receptor Fok1, and Toll-like receptor 2 are associated with extrapulmonary tuberculosis. **BMC Med Genet**. 2010;11:37. doi: 10.1186/1471-2350-11-37. PubMed PMID: 20196868; PMCID: PMC2837863.

59. Lange LA, Croteau-Chonka DC, Marvelle AF, Qin L, Gaulton KJ, Kuzawa CW, McDade TW, Wang Y, Li Y, Levy S, Borja JB, Lange EM, Adair LS, Mohlke KL. Genome-wide association study of homocysteine levels in Filipinos provides evidence for CPS1 in women and a stronger MTHFR effect in young adults. **Hum Mol Genet**. 2010;19(10):2050-2058. doi: 10.1093/hmg/ddq062. PubMed PMID: 20154341; PMCID: PMC2860887.

60. Otto EA, Hurd TW, Airik R, Chaki M, Zhou W, Stoetzel C, Patil SB, Levy S, Ghosh AK, Murga-Zamalloa CA, van Reeuwijk J, Letteboer SJ, Sang L, Giles RH, Liu Q, Coene KL, Estrada-Cuzcano A, Collin RW, McLaughlin HM, Held S, Kasanuki JM, Ramaswami G, Conte J, Lopez I, Washburn J, Macdonald J, Hu J, Yamashita Y, Maher ER, Guay-Woodford LM, Neumann HP, Obermuller N, Koenekoop RK, Bergmann C, Bei X, Lewis RA, Katsanis N, Lopes V, Williams DS, Lyons RH, Dang CV, Brito DA, Dias MB, Zhang X, Cavalcoli JD, Nurnberg G, Nurnberg P, Pierce EA, Jackson PK, Antignac C, Saunier S, Roepman R, Dollfus H, Khanna H, Hildebrandt F. Candidate exome capture identifies mutation of SDCCAG8 as the cause of a retinal-renal ciliopathy. **Nat Genet**. 2010;42(10):840-850. doi: 10.1038/ng.662. PubMed PMID: 20835237; PMCID: PMC2947620.

61. Tan L, Liu R, Lei S, Pan R, Yang T, Yan H, Pei Y, Yang F, Zhang F, Pan F, Zhang Y, Hu H, Levy S, Deng H. A genome-wide association analysis implicates SOX6 as a candidate gene for wrist bone mass. **Sci China Life Sci**. 2010;53(9):1065-1072. doi: 10.1007/s11427-010-4056-7. PubMed PMID: 21104366.

62. Xu B, Roos JL, Dexheimer P, Boone B, Plummer B, Levy S, Gogos JA, Karayiorgou M. Exome sequencing supports a de novo mutational paradigm for schizophrenia. **Nat Genet**. 2011;43(9):864-868. doi: 10.1038/ng.902. PubMed PMID: 21822266; PMCID: PMC3196550.

63. Cai Q, Long J, Lu W, Qu S, Wen W, Kang D, Lee JY, Chen K, Shen H, Shen CY, Sung H, Matsuo K, Haiman CA, Khoo US, Ren Z, Iwasaki M, Gu K, Xiang YB, Choi JY, Park SK, Zhang L, Hu Z, Wu PE, Noh DY, Tajima K, Henderson BE, Chan KY, Su F, Kasuga Y, Wang W, Cheng JR, Yoo KY, Lee JY, Zheng H, Liu Y, Shieh YL, Kim SW, Lee JW, Iwata H, Le Marchand L, Chan SY, Xie X, Tsugane S, Lee MH, Wang S, Li G, Levy S, Huang B, Shi J, Delahanty R, Zheng Y, Li C, Gao YT, Shu XO, Zheng W. Genome-wide association study identifies breast cancer risk variant at 10q21.2: results from the Asia Breast Cancer Consortium. **Hum Mol Genet**. 2011;20(24):4991-4999. doi: 10.1093/hmg/ddr405. PubMed PMID: 21908515; PMCID: PMC3221542.

64. Rademakers R, Baker M, Nicholson AM, Rutherford NJ, Finch N, Soto-Ortolaza A, Lash J, Wider C, Wojtas A, DeJesus-Hernandez M, Adamson J, Kouri N, Sundal C, Shuster EA, Aasly J, MacKenzie J, Roeber S, Kretzschmar HA, Boeve BF, Knopman DS, Petersen RC, Cairns NJ, Ghetti B, Spina S, Garbern J, Tselis AC, Uitti R, Das P, Van Gerpen JA, Meschia JF, Levy S, Broderick DF, Graff-Radford N, Ross OA, Miller BB, Swerdlow RH, Dickson DW, Wszolek ZK. Mutations in the colony stimulating factor 1 receptor (CSF1R)

gene cause hereditary diffuse leukoencephalopathy with spheroids. **Nat Genet**. 2011;44(2):200-205. doi: 10.1038/ng.1027. PubMed PMID: 22197934; PMCID: PMC3267847.

65.   Oki NO, Motsinger-Reif AA, Antas PR, Levy S, Holland SM, Sterling TR. Novel human genetic variants associated with extrapulmonary tuberculosis: a pilot genome wide association study. **BMC Res Notes**. 2011;4:28. doi: 10.1186/1756-0500-4-28. PubMed PMID: 21281516; PMCID: PMC3041678.

66.   Beane J, Vick J, Schembri F, Anderlind C, Gower A, Campbell J, Luo L, Zhang XH, Xiao J, Alekseyev YO, Wang S, Levy S, Massion PP, Lenburg M, Spira A. Characterizing the impact of smoking and lung cancer on the airway transcriptome using RNA-Seq. **Cancer Prev Res (Phila)**. 2011;4(6):803-817. doi: 10.1158/1940-6207.CAPR-11-0212. PubMed PMID: 21636547; PMCID: PMC3694393.

67.   Johansson S, Irgens H, Chudasama KK, Molnes J, Aerts J, Roque FS, Jonassen I, Levy S, Lima K, Knappskog PM, Bell GI, Molven A, Njolstad PR. Exome sequencing and genetic testing for MODY. **PLoS One**. 2012;7(5):e38050. doi: 10.1371/journal.pone.0038050. PubMed PMID: 22662265; PMCID: PMC3360646.

68.   Vilgelm A, Prasad N, Hawkins O, Levy S, Richmond A. Acquired resistance to Aurora A kinase inhibitor, Alisertib, in melanoma is associated with inhibition of tumor immune surveillance. **Pigment Cell & Melanoma Research**. 2012;25(6):894-895.

69.   Arrington CB, Bleyl SB, Matsunami N, Bonnell GD, Otterud BE, Nielsen DC, Stevens J, Levy S, Leppert MF, Bowles NE. Exome analysis of a family with pleiotropic congenital heart disease. **Circ Cardiovasc Genet**. 2012;5(2):175-182. doi: 10.1161/CIRCGENETICS.111.961797. PubMed PMID: 22337856; PMCID: PMC3329568.

70.   Neale BM, Kou Y, Liu L, Ma'ayan A, Samocha KE, Sabo A, Lin CF, Stevens C, Wang LS, Makarov V, Polak P, Yoon S, Maguire J, Crawford EL, Campbell NG, Geller ET, Valladares O, Schafer C, Liu H, Zhao T, Cai G, Lihm J, Dannenfelser R, Jabado O, Peralta Z, Nagaswamy U, Muzny D, Reid JG, Newsham I, Wu Y, Lewis L, Han Y, Voight BF, Lim E, Rossin E, Kirby A, Flannick J, Fromer M, Shakir K, Fennell T, Garimella K, Banks E, Poplin R, Gabriel S, DePristo M, Wimbish JR, Boone BE, Levy SE, Betancur C, Sunyaev S, Boerwinkle E, Buxbaum JD, Cook EH, Jr., Devlin B, Gibbs RA, Roeder K, Schellenberg GD, Sutcliffe JS, Daly MJ. Patterns and rates of exonic de novo mutations in autism spectrum disorders. **Nature**. 2012;485(7397):242-245. doi: 10.1038/nature11011. PubMed PMID: 22495311; PMCID: PMC3613847.

71.   Su Y, Vilgelm AE, Kelley MC, Hawkins OE, Liu Y, Boyd KL, Kantrow S, Splittgerber RC, Short SP, Sobolik T, Zaja-Milatovic S, Dahlman KB, Amiri KI, Jiang A, Lu P, Shyr Y, Stuart DD, Levy S, Sosman JA, Richmond A. RAF265 inhibits the growth of advanced human melanoma tumors. **Clin Cancer Res**. 2012;18(8):2184-2198. doi: 10.1158/1078-0432.CCR-11-1122. PubMed PMID: 22351689; PMCID: PMC3724517.

72.   Fiskerstrand T, Arshad N, Haukanes BI, Tronstad RR, Pham KD, Johansson S, Havik B, Tonder SL, Levy SE, Brackman D, Boman H, Biswas KH, Apold J, Hovdenak N, Visweswariah SS, Knappskog PM. Familial diarrhea syndrome caused by an activating GUCY2C mutation. **N Engl J Med**. 2012;366(17):1586-1595. doi: 10.1056/NEJMoa1110132. PubMed PMID: 22436048.

73.   Chaki M, Airik R, Ghosh AK, Giles RH, Chen R, Slaats GG, Wang H, Hurd TW, Zhou W, Cluckey A, Gee HY, Ramaswami G, Hong CJ, Hamilton BA, Cervenka I, Ganji RS, Bryja V, Arts HH, van Reeuwijk J, Oud MM, Letteboer SJ, Roepman R, Husson H, Ibraghimov-

Beskrovnaya O, Yasunaga T, Walz G, Eley L, Sayer JA, Schermer B, Liebau MC, Benzing T, Le Corre S, Drummond I, Janssen S, Allen SJ, Natarajan S, O'Toole JF, Attanasio M, Saunier S, Antignac C, Koenekoop RK, Ren H, Lopez I, Nayir A, Stoetzel C, Dollfus H, Massoudi R, Gleeson JG, Andreoli SP, Doherty DG, Lindstrad A, Golzio C, Katsanis N, Pape L, Abboud EB, Al-Rajhi AA, Lewis RA, Omran H, Lee EY, Wang S, Sekiguchi JM, Saunders R, Johnson CA, Garner E, Vanselow K, Andersen JS, Shlomai J, Nurnberg G, Nurnberg P, Levy S, Smogorzewska A, Otto EA, Hildebrandt F. Exome capture reveals ZNF423 and CEP164 mutations, linking renal ciliopathies to DNA damage response signaling. **Cell**. 2012;150(3):533-548. doi: 10.1016/j.cell.2012.06.028. PubMed PMID: 22863007; PMCID: PMC3433835.

74. Ding D, Scott NM, Thompson EE, Chaiworapongsa T, Torres R, Billstrand C, Murray K, Dexheimer PJ, Ismail M, Kay H, Levy S, Romero R, Lindheimer MD, Nicolae DL, Ober C. Increased protein-coding mutations in the mitochondrial genome of African American women with preeclampsia. **Reprod Sci**. 2012;19(12):1343-1351. doi: 10.1177/1933719112450337. PubMed PMID: 22902742; PMCID: PMC4046444.

75. Xu B, Ionita-Laza I, Roos JL, Boone B, Woodrick S, Sun Y, Levy S, Gogos JA, Karayiorgou M. De novo gene mutations highlight patterns of genetic and neural complexity in schizophrenia. **Nat Genet**. 2012;44(12):1365-1369. doi: 10.1038/ng.2446. PubMed PMID: 23042115; PMCID: PMC3556813.

76. Love C, Sun Z, Jima D, Li G, Zhang J, Miles R, Richards KL, Dunphy CH, Choi WW, Srivastava G, Lugar PL, Rizzieri DA, Lagoo AS, Bernal-Mizrachi L, Mann KP, Flowers CR, Naresh KN, Evens AM, Chadburn A, Gordon LI, Czader MB, Gill JI, Hsi ED, Greenough A, Moffitt AB, McKinney M, Banerjee A, Grubor V, Levy S, Dunson DB, Dave SS. The genetic landscape of mutations in Burkitt lymphoma. **Nat Genet**. 2012;44(12):1321-1325. doi: 10.1038/ng.2468. PubMed PMID: 23143597; PMCID: PMC3674561.

77. Zhou W, Otto EA, Cluckey A, Airik R, Hurd TW, Chaki M, Diaz K, Lach FP, Bennett GR, Gee HY, Ghosh AK, Natarajan S, Thongthip S, Veturi U, Allen SJ, Janssen S, Ramaswami G, Dixon J, Burkhalter F, Spoendlin M, Moch H, Mihatsch MJ, Verine J, Reade R, Soliman H, Godin M, Kiss D, Monga G, Mazzucco G, Amann K, Artunc F, Newland RC, Wiech T, Zschiedrich S, Huber TB, Friedl A, Slaats GG, Joles JA, Goldschmeding R, Washburn J, Giles RH, Levy S, Smogorzewska A, Hildebrandt F. FAN1 mutations cause karyomegalic interstitial nephritis, linking chronic kidney failure to defective DNA damage repair. **Nat Genet**. 2012;44(8):910-915. doi: 10.1038/ng.2347. PubMed PMID: 22772369; PMCID: PMC3412140.

78. Nam KT, O'Neal R, Lee YS, Lee YC, Coffey RJ, Goldenring JR. Gastric tumor development in Smad3-deficient mice initiates from forestomach/glandular transition zone along the lesser curvature. **Lab Invest**. 2012;92(6):883-895. doi: 10.1038/labinvest.2012.47. PubMed PMID: 22411066; PMCID: PMC3584162.

79. Chen XD, Xiong DH, Yang TL, Pei YF, Guo YF, Li J, Yang F, Pan F, Tan LJ, Yan H, Liu XG, Lei SF, Li X, Ning LL, Zhu XZ, Levy S, Kranzler HR, Farrer LA, Gelernter J, Recker RR, Deng HW. ANKRD7 and CYTL1 are novel risk genes for alcohol drinking behavior. **Chin Med J (Engl)**. 2012;125(6):1127-1134. PubMed PMID: 22613542; PMCID: PMC4174677.

80. Powell AE, Wang Y, Li Y, Poulin EJ, Means AL, Washington MK, Higginbotham JN, Juchheim A, Prasad N, Levy SE, Guo Y, Shyr Y, Aronow BJ, Haigis KM, Franklin JL, Coffey RJ. The pan-ErbB negative regulator Lrig1 is an intestinal stem cell marker that

functions as a tumor suppressor. **Cell**. 2012;149(1):146-158. doi: 10.1016/j.cell.2012.02.042. PubMed PMID: 22464327; PMCID: PMC3563328.

81.   Guo Y, Cai Q, Samuels DC, Ye F, Long J, Li CI, Winther JF, Tawn EJ, Stovall M, Lahteenmaki P, Malila N, Levy S, Shaffer C, Shyr Y, Shu XO, Boice JD, Jr. The use of next generation sequencing technology to study the effect of radiation therapy on mitochondrial DNA mutation. **Mutat Res**. 2012;744(2):154-160. doi: 10.1016/j.mrgentox.2012.02.006. PubMed PMID: 22387842; PMCID: PMC3354959.

82.   Consortium EP. An integrated encyclopedia of DNA elements in the human genome. **Nature**. 2012;489(7414):57-74. doi: 10.1038/nature11247. PubMed PMID: 22955616; PMCID: PMC3439153.

83.   Buehler D, Skelton S, Corpening J, Prasad N, Levy S, Clark T, Southard-Smith E. Differential expression of Phox2b marks distinct progenitor cell populations and facilitates analysis of regulatory pathways in enteric ontogeny. **Autonomic Neuroscience: Basic and Clinical**. 2013;177(2):297.

84.   Walter RB, Laszlo GS, Alonzo TA, Gerbing RB, Levy S, Fitzgibbon MP, Gudgeon CJ, Ries RE, Harrington KH, Raimondi SC, Hirsch BA, Gamis AS, M WM, Meshinchi S. Significance of expression of ITGA5 and its splice variants in acute myeloid leukemia: a report from the Children's Oncology Group. **Am J Hematol**. 2013;88(8):694-702. doi: 10.1002/ajh.23486. PubMed PMID: 23686445; PMCID: PMC3757130.

85.   Ramirez AH, Shaffer CM, Delaney JT, Sexton DP, Levy SE, Rieder MJ, Nickerson DA, George AL, Jr., Roden DM. Novel rare variants in congenital cardiac arrhythmia genes are frequent in drug-induced torsades de pointes. **Pharmacogenomics J**. 2013;13(4):325-329. doi: 10.1038/tpj.2012.14. PubMed PMID: 22584458; PMCID: PMC3422407.

86.   Gee HY, Saisawat P, Ashraf S, Hurd TW, Vega-Warner V, Fang H, Beck BB, Gribouval O, Zhou W, Diaz KA, Natarajan S, Wiggins RC, Lovric S, Chernin G, Schoeb DS, Ovunc B, Frishberg Y, Soliman NA, Fathy HM, Goebel H, Hoefele J, Weber LT, Innis JW, Faul C, Han Z, Washburn J, Antignac C, Levy S, Otto EA, Hildebrandt F. ARHGDIA mutations cause nephrotic syndrome via defective RHO GTPase signaling. **J Clin Invest**. 2013;123(8):3243-3253. doi: 10.1172/JCI69134. PubMed PMID: 23867502; PMCID: PMC3726174.

87.   Zariwala MA, Gee HY, Kurkowiak M, Al-Mutairi DA, Leigh MW, Hurd TW, Hjeij R, Dell SD, Chaki M, Dougherty GW, Adan M, Spear PC, Esteve-Rudd J, Loges NT, Rosenfeld M, Diaz KA, Olbrich H, Wolf WE, Sheridan E, Batten TF, Halbritter J, Porath JD, Kohl S, Lovric S, Hwang DY, Pittman JE, Burns KA, Ferkol TW, Sagel SD, Olivier KN, Morgan LC, Werner C, Raidt J, Pennekamp P, Sun Z, Zhou W, Airik R, Natarajan S, Allen SJ, Amirav I, Wieczorek D, Landwehr K, Nielsen K, Schwerk N, Sertic J, Kohler G, Washburn J, Levy S, Fan S, Koerner-Rettberg C, Amselem S, Williams DS, Mitchell BJ, Drummond IA, Otto EA, Omran H, Knowles MR, Hildebrandt F. ZMYND10 is mutated in primary ciliary dyskinesia and interacts with LRRC6. **Am J Hum Genet**. 2013;93(2):336-345. doi: 10.1016/j.ajhg.2013.06.007. PubMed PMID: 23891469; PMCID: PMC3738827.

88.   Ashraf S, Gee HY, Woerner S, Xie LX, Vega-Warner V, Lovric S, Fang H, Song X, Cattran DC, Avila-Casado C, Paterson AD, Nitschke P, Bole-Feysot C, Cochat P, Esteve-Rudd J, Haberberger B, Allen SJ, Zhou W, Airik R, Otto EA, Barua M, Al-Hamed MH, Kari JA, Evans J, Bierzynska A, Saleem MA, Bockenhauer D, Kleta R, El Desoky S, Hacihamdioglu DO, Gok F, Washburn J, Wiggins RC, Choi M, Lifton RP, Levy S, Han Z, Salviati L, Prokisch H, Williams DS, Pollak M, Clarke CF, Pei Y, Antignac C, Hildebrandt F. ADCK4 mutations promote steroid-resistant nephrotic syndrome through CoQ10 biosynthesis

disruption. **J Clin Invest**. 2013;123(12):5179-5189. doi: 10.1172/JCI69000. PubMed PMID: 24270420; PMCID: PMC3859425.

89.   Zhang J, Grubor V, Love CL, Banerjee A, Richards KL, Mieczkowski PA, Dunphy C, Choi W, Au WY, Srivastava G, Lugar PL, Rizzieri DA, Lagoo AS, Bernal-Mizrachi L, Mann KP, Flowers C, Naresh K, Evens A, Gordon LI, Czader M, Gill JI, Hsi ED, Liu Q, Fan A, Walsh K, Jima D, Smith LL, Johnson AJ, Byrd JC, Luftig MA, Ni T, Zhu J, Chadburn A, Levy S, Dunson D, Dave SS. Genetic heterogeneity of diffuse large B-cell lymphoma. **Proc Natl Acad Sci U S A**. 2013;110(4):1398-1403. doi: 10.1073/pnas.1205299110. PubMed PMID: 23292937; PMCID: PMC3557051.

90.   Chiu IM, Morimoto ET, Goodarzi H, Liao JT, O'Keeffe S, Phatnani HP, Muratet M, Carroll MC, Levy S, Tavazoie S, Myers RM, Maniatis T. A neurodegeneration-specific gene-expression signature of acutely isolated microglia from an amyotrophic lateral sclerosis mouse model. **Cell Rep**. 2013;4(2):385-401. doi: 10.1016/j.celrep.2013.06.018. PubMed PMID: 23850290; PMCID: PMC4272581.

91.   Pekow J, Dougherty U, Huang Y, Gometz E, Nathanson J, Cohen G, Levy S, Kocherginsky M, Venu N, Westerhoff M, Hart J, Noffsinger AE, Hanauer SB, Hurst RD, Fichera A, Joseph LJ, Liu Q, Bissonnette M. Gene signature distinguishes patients with chronic ulcerative colitis harboring remote neoplastic lesions. **Inflamm Bowel Dis**. 2013;19(3):461-470. doi: 10.1097/MIB.0b013e3182802bac. PubMed PMID: 23388545; PMCID: PMC3836269.

92.   Franklin JL, Rankin CR, Levy S, Snoddy JR, Zhang B, Washington MK, Thomson JM, Whitehead RH, Coffey RJ. Malignant transformation of colonic epithelial cells by a colon-derived long noncoding RNA. **Biochem Biophys Res Commun**. 2013;440(1):99-104. doi: 10.1016/j.bbrc.2013.09.040. PubMed PMID: 24045012; PMCID: PMC3875348.

93.   Ning MS, Kim AS, Prasad N, Levy SE, Zhang H, Andl T. Characterization of the Merkel Cell Carcinoma miRNome. **J Skin Cancer**. 2014;2014:289548. doi: 10.1155/2014/289548. PubMed PMID: 24627810; PMCID: PMC3929981.

94.   Royse KE, Zhi D, Conner MG, Clodfelder-Miller B, Srinivasasainagendra V, Vaughan LK, Skibola CF, Crossman DK, Levy S, Shrestha S. Differential Gene Expression Landscape of Co-Existing Cervical Pre-Cancer Lesions Using RNA-seq. **Front Oncol**. 2014;4:339. doi: 10.3389/fonc.2014.00339. PubMed PMID: 25505737; PMCID: PMC4244708.

95.   Gee HY, Otto EA, Hurd TW, Ashraf S, Chaki M, Cluckey A, Vega-Warner V, Saisawat P, Diaz KA, Fang H, Kohl S, Allen SJ, Airik R, Zhou W, Ramaswami G, Janssen S, Fu C, Innis JL, Weber S, Vester U, Davis EE, Katsanis N, Fathy HM, Jeck N, Klaus G, Nayir A, Rahim KA, Al Attrach I, Al Hassoun I, Ozturk S, Drozdz D, Helmchen U, O'Toole JF, Attanasio M, Lewis RA, Nurnberg G, Nurnberg P, Washburn J, MacDonald J, Innis JW, Levy S, Hildebrandt F. Whole-exome resequencing distinguishes cystic kidney diseases from phenocopies in renal ciliopathies. **Kidney Int**. 2014;85(4):880-887. doi: 10.1038/ki.2013.450. PubMed PMID: 24257694; PMCID: PMC3972265.

96.   Reinert RB, Cai Q, Hong JY, Plank JL, Aamodt K, Prasad N, Aramandla R, Dai C, Levy SE, Pozzi A, Labosky PA, Wright CV, Brissova M, Powers AC. Vascular endothelial growth factor coordinates islet innervation via vascular scaffolding. **Development**. 2014;141(7):1480-1491. doi: 10.1242/dev.098657. PubMed PMID: 24574008; PMCID: PMC3957372.

97.   Vrieze SI, Malone SM, Vaidyanathan U, Kwong A, Kang HM, Zhan X, Flickinger M, Irons D, Jun G, Locke AE, Pistis G, Porcu E, Levy S, Myers RM, Oetting W, McGue M, Abecasis G, Iacono WG. In search of rare variants: preliminary results from whole genome sequencing

of 1,325 individuals with psychophysiological endophenotypes. **Psychophysiology**. 2014;51(12):1309-1320. doi: 10.1111/psyp.12350. PubMed PMID: 25387710; PMCID: PMC4231480.

98. Gee HY, Ashraf S, Wan X, Vega-Warner V, Esteve-Rudd J, Lovric S, Fang H, Hurd TW, Sadowski CE, Allen SJ, Otto EA, Korkmaz E, Washburn J, Levy S, Williams DS, Bakkaloglu SA, Zolotnitskaya A, Ozaltin F, Zhou W, Hildebrandt F. Mutations in EMP2 cause childhood-onset nephrotic syndrome. **Am J Hum Genet**. 2014;94(6):884-890. doi: 10.1016/j.ajhg.2014.04.010. PubMed PMID: 24814193; PMCID: PMC4121470.

99. Brissova M, Aamodt K, Brahmachary P, Prasad N, Hong JY, Dai C, Mellati M, Shostak A, Poffenberger G, Aramandla R, Levy SE, Powers AC. Islet microenvironment, modulated by vascular endothelial growth factor-A signaling, promotes beta cell regeneration. **Cell Metab**. 2014;19(3):498-511. doi: 10.1016/j.cmet.2014.02.001. PubMed PMID: 24561261; PMCID: PMC4012856.

100. Zhang J, Jima D, Moffitt AB, Liu Q, Czader M, Hsi ED, Fedoriw Y, Dunphy CH, Richards KL, Gill JI, Sun Z, Love C, Scotland P, Lock E, Levy S, Hsu DS, Dunson D, Dave SS. The genomic landscape of mantle cell lymphoma is related to the epigenetically determined chromatin state of normal B cells. **Blood**. 2014;123(19):2988-2996. doi: 10.1182/blood-2013-07-517177. PubMed PMID: 24682267; PMCID: PMC4014841.

101. Campbell AG, Schwientek P, Vishnivetskaya T, Woyke T, Levy S, Beall CJ, Griffen A, Leys E, Podar M. Diversity and genomic insights into the uncultured Chloroflexi from the human microbiota. **Environ Microbiol**. 2014;16(9):2635-2643. doi: 10.1111/1462-2920.12461. PubMed PMID: 24738594; PMCID: PMC4149597.

102. Li S, Tighe SW, Nicolet CM, Grove D, Levy S, Farmerie W, Viale A, Wright C, Schweitzer PA, Gao Y, Kim D, Boland J, Hicks B, Kim R, Chhangawala S, Jafari N, Raghavachari N, Gandara J, Garcia-Reyero N, Hendrickson C, Roberson D, Rosenfeld J, Smith T, Underwood JG, Wang M, Zumbo P, Baldwin DA, Grills GS, Mason CE. Multi-platform assessment of transcriptome profiling using RNA-seq in the ABRF next-generation sequencing study. **Nat Biotechnol**. 2014;32(9):915-925. doi: 10.1038/nbt.2972. PubMed PMID: 25150835; PMCID: PMC4167418.

103. Picard M, Zhang J, Hancock S, Derbeneva O, Golhar R, Golik P, O'Hearn S, Levy S, Potluri P, Lvova M, Davila A, Lin CS, Perin JC, Rappaport EF, Hakonarson H, Trounce IA, Procaccio V, Wallace DC. Progressive increase in mtDNA 3243A>G heteroplasmy causes abrupt transcriptional reprogramming. **Proc Natl Acad Sci U S A**. 2014;111(38):E4033-4042. doi: 10.1073/pnas.1414028111. PubMed PMID: 25192935; PMCID: PMC4183335.

104. Vilgelm A, Johnson A, Prasad N, Yang J, Chen S-c, Ayers G, Pawlikowski J, Raman D, Sosman J, Kelley M. Therapy-induced senescence promotes a tumor suppressive immune microenvironment via Nfκb-mediated induction of Ccl5. **Pigment Cell & Melanoma Research**. 2015;28(6):820.

105. Hsu CH, Nguyen C, Yan C, Ries RE, Chen QR, Hu Y, Ostronoff F, Stirewalt DL, Komatsoulis G, Levy S, Meerzaman D, Meshinchi S. Transcriptome Profiling of Pediatric Core Binding Factor AML. **PLoS One**. 2015;10(9):e0138782. doi: 10.1371/journal.pone.0138782. PubMed PMID: 26397705; PMCID: PMC4580636.

106. Hoek KL, Samir P, Howard LM, Niu X, Prasad N, Galassie A, Liu Q, Allos TM, Floyd KA, Guo Y, Shyr Y, Levy SE, Joyce S, Edwards KM, Link AJ. A cell-based systems biology assessment of human blood to monitor immune responses after influenza vaccination.

PLoS One. 2015;10(2):e0118528. doi: 10.1371/journal.pone.0118528. PubMed PMID: 25706537; PMCID: PMC4338067.

107. Afshinnekoo E, Meydan C, Chowdhury S, Jaroudi D, Boyer C, Bernstein N, Maritz JM, Reeves D, Gandara J, Chhangawala S. Modern methods for delineating metagenomic complexity. **Cell systems**. 2015;1(1):6-7.

108. Strong KA, May T, Levy SA. In sickness and in health: context matters when considering potential benefits and risks of genome-wide sequencing. **Genet Med**. 2015;17(8):681-682. doi: 10.1038/gim.2015.85. PubMed PMID: 26240975.

109. Zhi D, Shendre A, Scherzer R, Irvin MR, Perry RT, Levy S, Arnett DK, Grunfeld C, Shrestha S. Deep sequencing of RYR3 gene identifies rare and common variants associated with increased carotid intima-media thickness (cIMT) in HIV-infected individuals. **J Hum Genet**. 2015;60(2):63-67. doi: 10.1038/jhg.2014.104. PubMed PMID: 25500725.

110. Ebarasi L, Ashraf S, Bierzynska A, Gee HY, McCarthy HJ, Lovric S, Sadowski CE, Pabst W, Vega-Warner V, Fang H, Koziell A, Simpson MA, Dursun I, Serdaroglu E, Levy S, Saleem MA, Hildebrandt F, Majumdar A. Defects of CRB2 cause steroid-resistant nephrotic syndrome. **Am J Hum Genet**. 2015;96(1):153-161. doi: 10.1016/j.ajhg.2014.11.014. PubMed PMID: 25557779; PMCID: PMC4289689.

111. Cha DJ, Franklin JL, Dou Y, Liu Q, Higginbotham JN, Demory Beckler M, Weaver AM, Vickers K, Prasad N, Levy S, Zhang B, Coffey RJ, Patton JG. KRAS-dependent sorting of miRNA to exosomes. **Elife**. 2015;4:e07197. doi: 10.7554/eLife.07197. PubMed PMID: 26132860; PMCID: PMC4510696.

112. Afshinnekoo E, Meydan C, Chowdhury S, Jaroudi D, Boyer C, Bernstein N, Maritz JM, Reeves D, Gandara J, Chhangawala S, Ahsanuddin S, Simmons A, Nessel T, Sundaresh B, Pereira E, Jorgensen E, Kolokotronis SO, Kirchberger N, Garcia I, Gandara D, Dhanraj S, Nawrin T, Saletore Y, Alexander N, Vijay P, Henaff EM, Zumbo P, Walsh M, O'Mullan GD, Tighe S, Dudley JT, Dunaif A, Ennis S, O'Halloran E, Magalhaes TR, Boone B, Jones AL, Muth TR, Paolantonio KS, Alter E, Schadt EE, Garbarino J, Prill RJ, Carlton JM, Levy S, Mason CE. Geospatial Resolution of Human and Bacterial Diversity with City-Scale Metagenomics. **Cell Syst**. 2015;1(1):72-87. doi: 10.1016/j.cels.2015.01.001. PubMed PMID: 26594662; PMCID: PMC4651444.

113. Gee HY, Zhang F, Ashraf S, Kohl S, Sadowski CE, Vega-Warner V, Zhou W, Lovric S, Fang H, Nettleton M, Zhu JY, Hoefele J, Weber LT, Podracka L, Boor A, Fehrenbach H, Innis JW, Washburn J, Levy S, Lifton RP, Otto EA, Han Z, Hildebrandt F. KANK deficiency leads to podocyte dysfunction and nephrotic syndrome. **J Clin Invest**. 2015;125(6):2375-2384. doi: 10.1172/JCI79504. PubMed PMID: 25961457; PMCID: PMC4497755.

114. Cirulli ET, Lasseigne BN, Petrovski S, Sapp PC, Dion PA, Leblond CS, Couthouis J, Lu YF, Wang Q, Krueger BJ, Ren Z, Keebler J, Han Y, Levy SE, Boone BE, Wimbish JR, Waite LL, Jones AL, Carulli JP, Day-Williams AG, Staropoli JF, Xin WW, Chesi A, Raphael AR, McKenna-Yasek D, Cady J, Vianney de Jong JM, Kenna KP, Smith BN, Topp S, Miller J, Gkazi A, Consortium FS, Al-Chalabi A, van den Berg LH, Veldink J, Silani V, Ticozzi N, Shaw CE, Baloh RH, Appel S, Simpson E, Lagier-Tourenne C, Pulst SM, Gibson S, Trojanowski JQ, Elman L, McCluskey L, Grossman M, Shneider NA, Chung WK, Ravits JM, Glass JD, Sims KB, Van Deerlin VM, Maniatis T, Hayes SD, Ordureau A, Swarup S, Landers J, Baas F, Allen AS, Bedlack RS, Harper JW, Gitler AD, Rouleau GA, Brown R, Harms MB, Cooper GM, Harris T, Myers RM, Goldstein DB. Exome sequencing in amyotrophic lateral sclerosis identifies risk genes and pathways. **Science**.

2015;347(6229):1436-1441. doi: 10.1126/science.aaa3650. PubMed PMID: 25700176; PMCID: PMC4437632.

115. Zea L, Prasad N, Levy SE, Stodieck L, Jones A, Shrestha S, Klaus D. A Molecular Genetic Basis Explaining Altered Bacterial Behavior in Space. **PLoS One**. 2016;11(11):e0164359. doi: 10.1371/journal.pone.0164359. PubMed PMID: 27806055; PMCID: PMC5091764.

116. McGrath K, McGrath L, Gray A, Osuolale O, Segata N, Fillo S, Iraola G, Zhou Y, Chang Y, Li Y. The Metagenomics and Metadesign of the Subways and Urban Biomes (MetaSUB) International Consortium Inaugural Meeting Report (vol 4, 24, 2016). **MICROBIOME**. 2016;4.

117. Levy SE, Myers RM. Advancements in next-generation sequencing. **Annual review of genomics and human genetics**. 2016;17:95-115.

118. Graham HT, Rotroff DM, Marvel SW, Buse JB, Havener TM, Wilson AG, Wagner MJ, Motsinger-Reif AA, Investigators AA. Incorporating Concomitant Medications into Genome-Wide Analyses for the Study of Complex Disease and Drug Response. **Frontiers in genetics**. 2016;7.

119. Alam SG, Zhang Q, Prasad N, Li Y, Chamala S, Kuchibhotla R, Kc B, Aggarwal V, Shrestha S, Jones AL, Levy SE, Roux KJ, Nickerson JA, Lele TP. The mammalian LINC complex regulates genome transcriptional responses to substrate rigidity. **Sci Rep**. 2016;6:38063. doi: 10.1038/srep38063. PubMed PMID: 27905489; PMCID: PMC5131312.

120. Grimes JA, Prasad N, Levy S, Cattley R, Lindley S, Boothe HW, Henderson RA, Smith BF. A comparison of microRNA expression profiles from splenic hemangiosarcoma, splenic nodular hyperplasia, and normal spleens of dogs. **BMC Vet Res**. 2016;12(1):272. doi: 10.1186/s12917-016-0903-5. PubMed PMID: 27912752; PMCID: PMC5135805.

121. Rahman KM, Camp ME, Prasad N, McNeel AK, Levy SE, Bartol FF, Bagnell CA. Age and Nursing Affect the Neonatal Porcine Uterine Transcriptome. **Biol Reprod**. 2016;94(2):46. doi: 10.1095/biolreprod.115.136150. PubMed PMID: 26632611.

122. Vilgelm AE, Johnson CA, Prasad N, Yang J, Chen SC, Ayers GD, Pawlikowski JS, Raman D, Sosman JA, Kelley M, Ecsedy JA, Shyr Y, Levy SE, Richmond A. Connecting the Dots: Therapy-Induced Senescence and a Tumor-Suppressive Immune Microenvironment. **J Natl Cancer Inst**. 2016;108(6):djv406. doi: 10.1093/jnci/djv406. PubMed PMID: 26719346; PMCID: PMC4849355.

123. Goes FS, Pirooznia M, Parla JS, Kramer M, Ghiban E, Mavruk S, Chen YC, Monson ET, Willour VL, Karchin R, Flickinger M, Locke AE, Levy SE, Scott LJ, Boehnke M, Stahl E, Moran JL, Hultman CM, Landen M, Purcell SM, Sklar P, Zandi PP, McCombie WR, Potash JB. Exome Sequencing of Familial Bipolar Disorder. **JAMA Psychiatry**. 2016;73(6):590-597. doi: 10.1001/jamapsychiatry.2016.0251. PubMed PMID: 27120077.

124. Dou Y, Cha DJ, Franklin JL, Higginbotham JN, Jeppesen DK, Weaver AM, Prasad N, Levy S, Coffey RJ, Patton JG, Zhang B. Circular RNAs are down-regulated in KRAS mutant colon cancer cells and can be transferred to exosomes. **Sci Rep**. 2016;6:37982. doi: 10.1038/srep37982. PubMed PMID: 27892494; PMCID: PMC5125100.

125. McCarthy S, Das S, Kretzschmar W, Delaneau O, Wood AR, Teumer A, Kang HM, Fuchsberger C, Danecek P, Sharp K, Luo Y, Sidore C, Kwong A, Timpson N, Koskinen S, Vrieze S, Scott LJ, Zhang H, Mahajan A, Veldink J, Peters U, Pato C, van Duijn CM, Gillies CE, Gandin I, Mezzavilla M, Gilly A, Cocca M, Traglia M, Angius A, Barrett JC, Boomsma D, Branham K, Breen G, Brummett CM, Busonero F, Campbell H, Chan A, Chen S, Chew E, Collins FS, Corbin LJ, Smith GD, Dedoussis G, Dorr M, Farmaki AE, Ferrucci L, Forer L,

Fraser RM, Gabriel S, Levy S, Groop L, Harrison T, Hattersley A, Holmen OL, Hveem K, Kretzler M, Lee JC, McGue M, Meitinger T, Melzer D, Min JL, Mohlke KL, Vincent JB, Nauck M, Nickerson D, Palotie A, Pato M, Pirastu N, McInnis M, Richards JB, Sala C, Salomaa V, Schlessinger D, Schoenherr S, Slagboom PE, Small K, Spector T, Stambolian D, Tuke M, Tuomilehto J, Van den Berg LH, Van Rheenen W, Volker U, Wijmenga C, Toniolo D, Zeggini E, Gasparini P, Sampson MG, Wilson JF, Frayling T, de Bakker PI, Swertz MA, McCarroll S, Kooperberg C, Dekker A, Altshuler D, Willer C, Iacono W, Ripatti S, Soranzo N, Walter K, Swaroop A, Cucca F, Anderson CA, Myers RM, Boehnke M, McCarthy MI, Durbin R, Haplotype Reference C. A reference panel of 64,976 haplotypes for genotype imputation. **Nat Genet**. 2016;48(10):1279-1283. doi: 10.1038/ng.3643. PubMed PMID: 27548312; PMCID: PMC5388176.

126. Das S, Forer L, Schonherr S, Sidore C, Locke AE, Kwong A, Vrieze SI, Chew EY, Levy S, McGue M, Schlessinger D, Stambolian D, Loh PR, Iacono WG, Swaroop A, Scott LJ, Cucca F, Kronenberg F, Boehnke M, Abecasis GR, Fuchsberger C. Next-generation genotype imputation service and methods. **Nat Genet**. 2016;48(10):1284-1287. doi: 10.1038/ng.3656. PubMed PMID: 27571263; PMCID: PMC5157836.

127. Mason C, McIntyre A, Ounit R, Afshinnekoo E, Prill R, Henaff E, Alexander N, Minot S, Danko D, Foox J. Comprehensive Benchmarking and Ensemble Approaches for Metagenomic Classifiers. **bioRxiv**. 2017:156919.

128. Howard LM, Hoek KL, Goll JB, Samir P, Galassie A, Allos TM, Niu X, Gordy LE, Creech CB, Prasad N, Jensen TL, Hill H, Levy SE, Joyce S, Link AJ, Edwards KM. Cell-Based Systems Biology Analysis of Human AS03-Adjuvanted H5N1 Avian Influenza Vaccine Responses: A Phase I Randomized Controlled Trial. **PLoS One**. 2017;12(1):e0167488. doi: 10.1371/journal.pone.0167488. PubMed PMID: 28099485; PMCID: PMC5242433.

129. Carlson J, Scott LJ, Locke AE, Flickinger M, Levy S, Myers RM, Boehnke M, Kang HM, Li JZ, Zöllner S. Extremely rare variants reveal patterns of germline mutation rate heterogeneity in humans. **bioRxiv**. 2017:108290.

130. Johansson BB, Irgens HU, Molnes J, Sztromwasser P, Aukrust I, Juliusson PB, Sovik O, Levy S, Skrivarhaug T, Joner G, Molven A, Johansson S, Njolstad PR. Targeted next-generation sequencing reveals MODY in up to 6.5% of antibody-negative diabetes cases listed in the Norwegian Childhood Diabetes Registry. **Diabetologia**. 2017;60(4):625-635. doi: 10.1007/s00125-016-4167-1. PubMed PMID: 27913849.

131. Mason CE, Afshinnekoo E, Tighe S, Wu S, Levy S. International Standards for Genomes, Transcriptomes, and Metagenomes. **J Biomol Tech**. 2017;28(1):8-18. doi: 10.7171/jbt.17-2801-006. PubMed PMID: 28337071; PMCID: PMC5359768.

132. McKinney M, Moffitt AB, Gaulard P, Travert M, De Leval L, Nicolae A, Raffeld M, Jaffe ES, Pittaluga S, Xi L, Heavican T, Iqbal J, Belhadj K, Delfau-Larue MH, Fataccioli V, Czader MB, Lossos IS, Chapman-Fredricks JR, Richards KL, Fedoriw Y, Ondrejka SL, Hsi ED, Low L, Weisenburger D, Chan WC, Mehta-Shah N, Horwitz S, Bernal-Mizrachi L, Flowers CR, Beaven AW, Parihar M, Baseggio L, Parrens M, Moreau A, Sujobert P, Pilichowska M, Evens AM, Chadburn A, Au-Yeung RK, Srivastava G, Choi WW, Goodlad JR, Aurer I, Basic-Kinda S, Gascoyne RD, Davis NS, Li G, Zhang J, Rajagopalan D, Reddy A, Love C, Levy S, Zhuang Y, Datta J, Dunson DB, Dave SS. The Genetic Basis of Hepatosplenic T-cell Lymphoma. **Cancer Discov**. 2017;7(4):369-379. doi: 10.1158/2159-8290.CD-16-0330. PubMed PMID: 28122867; PMCID: PMC5402251.

133. Tighe S, Afshinnekoo E, Rock TM, McGrath K, Alexander N, McIntyre A, Ahsanuddin S, Bezdan D, Green SJ, Joye S, Stewart Johnson S, Baldwin DA, Bivens N, Ajami N,

Carmical JR, Herriott IC, Colwell R, Donia M, Foox J, Greenfield N, Hunter T, Hoffman J, Hyman J, Jorgensen E, Krawczyk D, Lee J, Levy S, Garcia-Reyero N, Settles M, Thomas K, Gomez F, Schriml L, Kyrpides N, Zaikova E, Penterman J, Mason CE. Genomic Methods and Microbiological Technologies for Profiling Novel and Extreme Environments for the Extreme Microbiome Project (XMP). **J Biomol Tech**. 2017;28(1):31-39. doi: 10.7171/jbt.17-2801-004. PubMed PMID: 28337070; PMCID: PMC5345951.

134. Gosline SJ, Weinberg H, Knight P, Yu T, Guo X, Prasad N, Jones A, Shrestha S, Boone B, Levy SE, La Rosa S, Guinney J, Bakker A. A high-throughput molecular data resource for cutaneous neurofibromas. **Sci Data**. 2017;4:170045. doi: 10.1038/sdata.2017.45. PubMed PMID: 28398289; PMCID: PMC5387919.

135. Chao HT, Davids M, Burke E, Pappas JG, Rosenfeld JA, McCarty AJ, Davis T, Wolfe L, Toro C, Tifft C, Xia F, Stong N, Johnson TK, Warr CG, Undiagnosed Diseases N, Yamamoto S, Adams DR, Markello TC, Gahl WA, Bellen HJ, Wangler MF, Malicdan MC. A Syndromic Neurodevelopmental Disorder Caused by De Novo Variants in EBF3. **Am J Hum Genet**. 2017;100(1):128-137. doi: 10.1016/j.ajhg.2016.11.018. PubMed PMID: 28017372; PMCID: PMC5223093.

136. McDaniel JM, Varley KE, Gertz J, Savic DS, Roberts BS, Bailey SK, Shevde LA, Ramaker RC, Lasseigne BN, Kirby MK, Newberry KM, Partridge EC, Jones AL, Boone B, Levy SE, Oliver PG, Sexton KC, Grizzle WE, Forero A, Buchsbaum DJ, Cooper SJ, Myers RM. Genomic regulation of invasion by STAT3 in triple negative breast cancer. **Oncotarget**. 2017;8(5):8226-8238. doi: 10.18632/oncotarget.14153. PubMed PMID: 28030809; PMCID: PMC5352396.

137. Dean ED, Li M, Prasad N, Wisniewski SN, Von Deylen A, Spaeth J, Maddison L, Botros A, Sedgeman LR, Bozadjieva N, Ilkayeva O, Coldren A, Poffenberger G, Shostak A, Semich MC, Aamodt KI, Phillips N, Yan H, Bernal-Mizrachi E, Corbin JD, Vickers KC, Levy SE, Dai C, Newgard C, Gu W, Stein R, Chen W, Powers AC. Interrupted Glucagon Signaling Reveals Hepatic alpha Cell Axis and Role for L-Glutamine in alpha Cell Proliferation. **Cell Metab**. 2017;25(6):1362-1373 e1365. doi: 10.1016/j.cmet.2017.05.011. PubMed PMID: 28591638.

138. Steffen MM, Davis TW, McKay RML, Bullerjahn GS, Krausfeldt LE, Stough JMA, Neitzey ML, Gilbert NE, Boyer GL, Johengen TH, Gossiaux DC, Burtner AM, Palladino D, Rowe MD, Dick GJ, Meyer KA, Levy S, Boone BE, Stumpf RP, Wynne TT, Zimba PV, Gutierrez D, Wilhelm SW. Ecophysiological Examination of the Lake Erie Microcystis Bloom in 2014: Linkages between Biology and the Water Supply Shutdown of Toledo, OH. **Environ Sci Technol**. 2017;51(12):6745-6755. doi: 10.1021/acs.est.7b00856. PubMed PMID: 28535339.

139. Luo W, Galvan DL, Woodard LE, Dorset D, Levy S, Wilson MH. Comparative analysis of chimeric ZFP-, TALE- and Cas9-piggyBac transposases for integration into a single locus in human cells. **Nucleic Acids Res**. 2017. doi: 10.1093/nar/gkx572. PubMed PMID: 28666380.

140. Moffitt AB, Ondrejka SL, McKinney M, Rempel RE, Goodlad JR, Teh CH, Leppa S, Mannisto S, Kovanen PE, Tse E, Au-Yeung RKH, Kwong YL, Srivastava G, Iqbal J, Yu J, Naresh K, Villa D, Gascoyne RD, Said J, Czader MB, Chadburn A, Richards KL, Rajagopalan D, Davis NS, Smith EC, Palus BC, Tzeng TJ, Healy JA, Lugar PL, Datta J, Love C, Levy S, Dunson DB, Zhuang Y, Hsi ED, Dave SS. Enteropathy-associated T cell lymphoma subtypes are characterized by loss of function of SETD2. **J Exp Med**.

2017;214(5):1371-1386. doi: 10.1084/jem.20160894. PubMed PMID: 28424246; PMCID: PMC5413324.

141. Dai C, Hang Y, Shostak A, Poffenberger G, Hart N, Prasad N, Phillips N, Levy SE, Greiner DL, Shultz LD, Bottino R, Kim SK, Powers AC. Age-dependent human beta cell proliferation induced by glucagon-like peptide 1 and calcineurin signaling. **J Clin Invest**. 2017;127(10):3835-3844. doi: 10.1172/JCI91761. PubMed PMID: 28920919; PMCID: PMC5617654.

142. Reddy A, Zhang J, Davis NS, Moffitt AB, Love CL, Waldrop A, Leppa S, Pasanen A, Meriranta L, Karjalainen-Lindsberg ML, Norgaard P, Pedersen M, Gang AO, Hogdall E, Heavican TB, Lone W, Iqbal J, Qin Q, Li G, Kim SY, Healy J, Richards KL, Fedoriw Y, Bernal-Mizrachi L, Koff JL, Staton AD, Flowers CR, Paltiel O, Goldschmidt N, Calaminici M, Clear A, Gribben J, Nguyen E, Czader MB, Ondrejka SL, Collie A, Hsi ED, Tse E, Au-Yeung RKH, Kwong YL, Srivastava G, Choi WWL, Evens AM, Pilichowska M, Sengar M, Reddy N, Li S, Chadburn A, Gordon LI, Jaffe ES, Levy S, Rempel R, Tzeng T, Happ LE, Dave T, Rajagopalan D, Datta J, Dunson DB, Dave SS. Genetic and Functional Drivers of Diffuse Large B Cell Lymphoma. **Cell**. 2017;171(2):481-494 e415. doi: 10.1016/j.cell.2017.09.027. PubMed PMID: 28985567; PMCID: PMC5659841.

143. McIntyre ABR, Ounit R, Afshinnekoo E, Prill RJ, Henaff E, Alexander N, Minot SS, Danko D, Foox J, Ahsanuddin S, Tighe S, Hasan NA, Subramanian P, Moffat K, Levy S, Lonardi S, Greenfield N, Colwell RR, Rosen GL, Mason CE. Comprehensive benchmarking and ensemble approaches for metagenomic classifiers. **Genome Biol**. 2017;18(1):182. doi: 10.1186/s13059-017-1299-7. PubMed PMID: 28934964; PMCID: PMC5609029.

144. O'Hara NB, Reed HJ, Afshinnekoo E, Harvin D, Caplan N, Rosen G, Frye B, Woloszynek S, Ounit R, Levy S, Butler E, Mason CE. Metagenomic characterization of ambulances across the USA. **Microbiome**. 2017;5(1):125. doi: 10.1186/s40168-017-0339-6. PubMed PMID: 28938903; PMCID: PMC5610413.

145. Aunins TR, Erickson KE, Prasad N, Levy SE, Jones A, Shrestha S, Mastracchio R, Stodieck L, Klaus D, Zea L, Chatterjee A. Spaceflight Modifies Escherichia coli Gene Expression in Response to Antibiotic Exposure and Reveals Role of Oxidative Stress Response. **Front Microbiol**. 2018;9:310. doi: 10.3389/fmicb.2018.00310. PubMed PMID: 29615983; PMCID: PMC5865062.

146. Thompson ML, Finnila CR, Bowling KM, Brothers KB, Neu MB, Amaral MD, Hiatt SM, East KM, Gray DE, Lawlor JMJ, Kelley WV, Lose EJ, Rich CA, Simmons S, Levy SE, Myers RM, Barsh GS, Bebin EM, Cooper GM. Genomic sequencing identifies secondary findings in a cohort of parent study participants. **Genet Med**. 2018. doi: 10.1038/gim.2018.53. PubMed PMID: 29790872.

147. Marcogliese PC, Shashi V, Spillmann RC, Stong N, Rosenfeld JA, Koenig MK, Martinez-Agosto JA, Herzog M, Chen AH, Dickson PI, Lin HJ, Vera MU, Salamon N, Graham JM, Jr., Ortiz D, Infante E, Steyaert W, Dermaut B, Poppe B, Chung HL, Zuo Z, Lee PT, Kanca O, Xia F, Yang Y, Smith EC, Jasien J, Kansagra S, Spiridigliozzi G, El-Dairi M, Lark R, Riley K, Koeberl DD, Golden-Grant K, Program for Undiagnosed D, Undiagnosed Diseases N, Yamamoto S, Wangler MF, Mirzaa G, Hemelsoet D, Lee B, Nelson SF, Goldstein DB, Bellen HJ, Pena LDM. IRF2BPL Is Associated with Neurological Phenotypes. **Am J Hum Genet**. 2018;103(2):245-260. doi: 10.1016/j.ajhg.2018.07.006. PubMed PMID: 30057031; PMCID: PMC6081494.

148. Schlegel C, Lapierre LA, Weis VG, Williams JA, Kaji I, Pinzon-Guzman C, Prasad N, Boone B, Jones A, Correa H, Levy SE, Han X, Wang M, Thomsen K, Acra S, Goldenring JR.

Reversible deficits in apical transporter trafficking associated with deficiency in diacylglycerol acyltransferase. **Traffic**. 2018. doi: 10.1111/tra.12608. PubMed PMID: 30095213.

149. Schlegel C, Lapierre LA, Weis VG, Williams JA, Kaji I, Pinzon-Guzman C, Prasad N, Boone B, Jones A, Correa H, Levy SE, Han X, Wang M, Thomsen K, Acra S, Goldenring JR. Reversible deficits in apical transporter trafficking associated with DGAT1 deficiency. **Traffic**. 2018. doi: 10.1111/tra.12608. PubMed PMID: 30095213.

150. Keele GR, Prokop JW, He H, Holl K, Littrell J, Deal A, Francic S, Cui L, Gatti DM, Broman KW, Tschannen M, Tsaih SW, Zagloul M, Kim Y, Baur B, Fox J, Robinson M, Levy S, Flister MJ, Mott R, Valdar W, Solberg Woods LC. Genetic Fine-Mapping and Identification of Candidate Genes and Variants for Adiposity Traits in Outbred Rats. **Obesity (Silver Spring)**. 2018;26(1):213-222. doi: 10.1002/oby.22075. PubMed PMID: 29193816; PMCID: PMC5740008.

151. Hiatt SM, Amaral MD, Bowling KM, Finnila CR, Thompson ML, Gray DE, Lawlor JMJ, Cochran JN, Bebin EM, Brothers KB, East KM, Kelley WV, Lamb NE, Levy SE, Lose EJ, Neu MB, Rich CA, Simmons S, Myers RM, Barsh GS, Cooper GM. Systematic reanalysis of genomic data improves quality of variant interpretation. **Clin Genet**. 2018;94(1):174-178. doi: 10.1111/cge.13259. PubMed PMID: 29652076; PMCID: PMC5995667.

152. Weiss H, Hertzberg VS, Dupont C, Espinoza JL, Levy S, Nelson K, Norris S, FlyHealthy Research T. The Airplane Cabin Microbiome. **Microb Ecol**. 2018. doi: 10.1007/s00248-018-1191-3. PubMed PMID: 29876609.

153. Bipolar D, Schizophrenia Working Group of the Psychiatric Genomics Consortium. Electronic address drve, Bipolar D, Schizophrenia Working Group of the Psychiatric Genomics C. Genomic Dissection of Bipolar Disorder and Schizophrenia, Including 28 Subphenotypes. **Cell**. 2018;173(7):1705-1715 e1716. doi: 10.1016/j.cell.2018.05.046. PubMed PMID: 29906448.

154. Shashi V, Schoch K, Spillmann R, Cope H, Tan QK, Walley N, Pena L, McConkie-Rosell A, Jiang YH, Stong N, Need AC, Goldstein DB, Undiagnosed Diseases N. A comprehensive iterative approach is highly effective in diagnosing individuals who are exome negative. **Genet Med**. 2018. doi: 10.1038/s41436-018-0044-2. PubMed PMID: 29907797.

155. Olahova M, Yoon WH, Thompson K, Jangam S, Fernandez L, Davidson JM, Kyle JE, Grove ME, Fisk DG, Kohler JN, Holmes M, Dries AM, Huang Y, Zhao C, Contrepois K, Zappala Z, Fresard L, Waggott D, Zink EM, Kim YM, Heyman HM, Stratton KG, Webb-Robertson BM, Undiagnosed Diseases N, Snyder M, Merker JD, Montgomery SB, Fisher PG, Feichtinger RG, Mayr JA, Hall J, Barbosa IA, Simpson MA, Deshpande C, Waters KM, Koeller DM, Metz TO, Morris AA, Schelley S, Cowan T, Friederich MW, McFarland R, Van Hove JLK, Enns GM, Yamamoto S, Ashley EA, Wangler MF, Taylor RW, Bellen HJ, Bernstein JA, Wheeler MT. Biallelic Mutations in ATP5F1D, which Encodes a Subunit of ATP Synthase, Cause a Metabolic Disorder. **Am J Hum Genet**. 2018;102(3):494-504. doi: 10.1016/j.ajhg.2018.01.020. PubMed PMID: 29478781.

156. Brissova M, Haliyur R, Saunders D, Shrestha S, Dai C, Blodgett DM, Bottino R, Campbell-Thompson M, Aramandla R, Poffenberger G, Lindner J, Pan FC, von Herrath MG, Greiner DL, Shultz LD, Sanyoura M, Philipson LH, Atkinson M, Harlan DM, Levy SE, Prasad N, Stein R, Powers AC. alpha Cell Function and Gene Expression Are Compromised in Type 1 Diabetes. **Cell Rep**. 2018;22(10):2667-2676. doi: 10.1016/j.celrep.2018.02.032. PubMed PMID: 29514095.

157. Nicolas A, Kenna KP, Renton AE, Ticozzi N, Faghri F, Chia R, Dominov JA, Kenna BJ, Nalls MA, Keagle P, Rivera AM, van Rheenen W, Murphy NA, van Vugt J, Geiger JT, Van der Spek RA, Pliner HA, Shankaracharya, Smith BN, Marangi G, Topp SD, Abramzon Y, Gkazi AS, Eicher JD, Kenna A, Consortium I, Mora G, Calvo A, Mazzini L, Riva N, Mandrioli J, Caponnetto C, Battistini S, Volanti P, La Bella V, Conforti FL, Borghero G, Messina S, Simone IL, Trojsi F, Salvi F, Logullo FO, D'Alfonso S, Corrado L, Capasso M, Ferrucci L, Genomic Translation for ALSCC, Moreno CAM, Kamalakaran S, Goldstein DB, Consortium ALSS, Gitler AD, Harris T, Myers RM, Consortium NA, Phatnani H, Musunuri RL, Evani US, Abhyankar A, Zody MC, Answer ALSF, Kaye J, Finkbeiner S, Wyman SK, LeNail A, Lima L, Fraenkel E, Svendsen CN, Thompson LM, Van Eyk JE, Berry JD, Miller TM, Kolb SJ, Cudkowicz M, Baxi E, Clinical Research in ALS, Related Disorders for Therapeutic Development C, Benatar M, Taylor JP, Rampersaud E, Wu G, Wuu J, Consortium S, Lauria G, Verde F, Fogh I, Tiloca C, Comi GP, Soraru G, Cereda C, French ALSC, Corcia P, Laaksovirta H, Myllykangas L, Jansson L, Valori M, Ealing J, Hamdalla H, Rollinson S, Pickering-Brown S, Orrell RW, Sidle KC, Malaspina A, Hardy J, Singleton AB, Johnson JO, Arepalli S, Sapp PC, McKenna-Yasek D, Polak M, Asress S, Al-Sarraj S, King A, Troakes C, Vance C, de Belleroche J, Baas F, Ten Asbroek A, Munoz-Blanco JL, Hernandez DG, Ding J, Gibbs JR, Scholz SW, Floeter MK, Campbell RH, Landi F, Bowser R, Pulst SM, Ravits JM, MacGowan DJL, Kirby J, Pioro EP, Pamphlett R, Broach J, Gerhard G, Dunckley TL, Brady CB, Kowall NW, Troncoso JC, Le Ber I, Mouzat K, Lumbroso S, Heiman-Patterson TD, Kamel F, Van Den Bosch L, Baloh RH, Strom TM, Meitinger T, Shatunov A, Van Eijk KR, de Carvalho M, Kooyman M, Middelkoop B, Moisse M, McLaughlin RL, Van Es MA, Weber M, Boylan KB, Van Blitterswijk M, Rademakers R, Morrison KE, Basak AN, Mora JS, Drory VE, Shaw PJ, Turner MR, Talbot K, Hardiman O, Williams KL, Fifita JA, Nicholson GA, Blair IP, Rouleau GA, Esteban-Perez J, Garcia-Redondo A, Al-Chalabi A, Project Min EALSSC, Rogaeva E, Zinman L, Ostrow LW, Maragakis NJ, Rothstein JD, Simmons Z, Cooper-Knock J, Brice A, Goutman SA, Feldman EL, Gibson SB, Taroni F, Ratti A, Gellera C, Van Damme P, Robberecht W, Fratta P, Sabatelli M, Lunetta C, Ludolph AC, Andersen PM, Weishaupt JH, Camu W, Trojanowski JQ, Van Deerlin VM, Brown RH, Jr., van den Berg LH, Veldink JH, Harms MB, Glass JD, Stone DJ, Tienari P, Silani V, Chio A, Shaw CE, Traynor BJ, Landers JE. Genome-wide Analyses Identify KIF5A as a Novel ALS Gene. **Neuron**. 2018;97(6):1268-1283 e1266. doi: 10.1016/j.neuron.2018.02.027. PubMed PMID: 29566793; PMCID: PMC5867896.

158. Petersen CP, Meyer AR, De Salvo C, Choi E, Schlegel C, Petersen A, Engevik AC, Prasad N, Levy SE, Peebles RS, Pizarro TT, Goldenring JR. A signalling cascade of IL-33 to IL-13 regulates metaplasia in the mouse stomach. **Gut**. 2018;67(5):805-817. doi: 10.1136/gutjnl-2016-312779. PubMed PMID: 28196875; PMCID: PMC5681443.

159. Liu N, Schoch K, Luo X, Pena LDM, Bhavana VH, Kukolich MK, Stringer S, Powis Z, Radtke K, Mroske C, Deak KL, McDonald MT, McConkie-Rosell A, Markert ML, Kranz PG, Stong N, Need AC, Bick D, Amaral MD, Worthey EA, Levy S, Undiagnosed Diseases N, Wangler MF, Bellen HJ, Shashi V, Yamamoto S. Functional variants in TBX2 are associated with a syndromic cardiovascular and skeletal developmental disorder. **Hum Mol Genet**. 2018. doi: 10.1093/hmg/ddy146. PubMed PMID: 29726930.

160. Selvan N, George S, Serajee FJ, Shaw M, Hobson L, Kalscheuer VM, Prasad N, Levy SE, Taylor J, Afitmos S, Schwartz CE, Huq AM, Gecz J, Wells L. O-GlcNAc transferase missense mutations linked to X-linked intellectual disability deregulate genes involved in cell fate determination and signaling. **J Biol Chem**. 2018. doi: 10.1074/jbc.RA118.002583. PubMed PMID: 29769320.

161. Wang Z, Wilson CL, Easton J, Thrasher A, Mulder H, Liu Q, Hedges DJ, Wang S, Rusch MC, Edmonson MN, Levy S, Lanctot JQ, Caron E, Shelton K, Currie K, Lear M, Patel A, Rosencrance C, Shao Y, Vadodaria B, Yergeau D, Sapkota Y, Brooke RJ, Moon W, Rampersaud E, Ma X, Chang TC, Rice SV, Pepper C, Zhou X, Chen X, Chen W, Jones A, Boone B, Ehrhardt MJ, Krasin MJ, Howell RM, Phillips NS, Lewis C, Srivastava D, Pui CH, Kesserwan CA, Wu G, Nichols KE, Downing JR, Hudson MM, Yasui Y, Robison LL, Zhang J. Genetic Risk for Subsequent Neoplasms Among Long-Term Survivors of Childhood Cancer. **J Clin Oncol**. 2018:JCO2018778589. doi: 10.1200/JCO.2018.77.8589. PubMed PMID: 29847298.

162. Carlson J, Locke AE, Flickinger M, Zawistowski M, Levy S, Myers RM, Boehnke M, Kang HM, Scott LJ, Li JZ, Zollner S, Bipolar Sequencing Consortium. Extremely rare variants reveal patterns of germline mutation rate heterogeneity in humans. **Nat Commun**. 2018;9(1):3753. doi: 10.1038/s41467-018-05936-5. PubMed PMID: 30218074; PMCID: PMC6138700.

Exhibit B

MATERIALS AND DATA CONSIDERED

Literature

Acencio, Milena M. P., Evaldo Marchi, Lisete R. Teixeira, Bruna Rocha Silva, Juliana Sanchez Silva, Carlos Sergio Rocha Silva, Vanessa Adelia Alvarenga, Leila Antonangelo, Francisco Suso Vargas, and Vera Luiza Capelozzi. "Talc Particles and Pleural Mesothelium Interface Modulate Apoptosis and Inflammation." *Pathology* 46, no. S2 (2014): S76.

Acheson, E D, M J Gardner, E C Pippard, and L P Grime. "Mortality of Two Groups of Women Who Manufactured Gas Masks from Chrysotile and Crocidolite Asbestos: A 40-Year Follow-Up." *British Journal of Industrial Medicine* 39, no. 4 (November 1982): 344–48.

Adami, H.O., Hsieh, C.C., Lambe, M., Trichopoulos, D., Leon, D., Persson, I., Ekbom, A., and Janson, P.O. (1994). Parity, age at first childbirth, and risk of ovarian cancer. Lancet *344*, 1250-1254.

Agic, A., Xu, H., Finas, D., Banz, C., Diedrich, K., and Hornung, D. (2006). Is endometriosis associated with systemic subclinical inflammation? Gynecol Obstet Invest *62*, 139-147.

Akhtar, Mohd Javed, Maqusood Ahamed, M.A. Majeed Khan, Salman A. Alrokayan, Iqbal Ahmad, and Sudhir Kumar. "Cytotoxicity and Apoptosis Induction by Nanoscale Talc Particles from Two Different Geographical Regions in Human Lung Epithelial Cells." *Environmental Toxicology* 29 (2014): 394–406. https://doi.org/10.1002/tox.21766 .

Akhtar, Mohd Javed, Sudhir Kumar, Ramesh Chandra Murthy, Mohd Ashquin, Mohd Imran Khan, Govil Patil, and Iqbal Ahmad. "The Primary Role of Iron-Mediated Lipid Peroxidation in the Differential Cytotoxicity Caused by Two Varieties of Talc Nanoparticles on A549 Cells and Lipid Peroxidation Inhibitory Effect Exerted by Ascorbic Acid." *Toxicology in Vitro: An International Journal Published in Association with BIBRA* 24, no. 4 (June 2010): 1139–47. https://doi.org/10.1016/j.tiv.2010.03.002.

Albu, Cristina-Crenguta. "The Genetics of Ovarian Cancer." *SMGroup*, (November 3, 2017).

Arellano-Orden, Elena, Auxiliadora Romero-Falcon, Jose Martin Juan, Manuel Ocana Jurado, Francisco Rodriguez-Panadero, and Ana Montes-Worboys. "Small Particle-Size Talc Is Associated with Poor Outcome and Increased Inflammation in Thoracoscopic Pleurodesis." *Respiration* 86 (2013): 201–9. https://doi.org/10.1159/000342042.

"ATSDR - Toxicological Profile: Asbestos." Accessed August 16, 2018. https://www.atsdr.cdc.gov/toxprofiles/tp.asp?id=30&tid=4.

Aust, A.E., Cook, P.M., and Dodson, R.F. Morphological and chemical mechanisms of elongated mineral particle toxicities.   J Toxicol and Environ Health, Part B.   (2011) 14:40-75.

Balkwill, F., and Mantovani, A. (2001). Inflammation and cancer: back to Virchow? Lancet *357*, 539-545.

Banerjee, S., and Kaye, S.B. (2013). New strategies in the treatment of ovarian cancer: current clinical perspectives and future potential. Clin Cancer Res *19*, 961-968.

Barnes, D.R., and Antoniou, A.C. (2012). Unravelling modifiers of breast and ovarian cancer risk for BRCA1 and BRCA2 mutation carriers: update on genetic modifiers. J Intern Med *271*, 331-343.

Begg, Melissa D., and Dana March. "Cause and Association: Missing the Forest for the Trees." *American Journal of Public Health* 108, No. 5 (May 2018): 620–620.

Belotte, J., Fletcher, N.M., Saed, M.G., Abusamaan, M.S., Dyson, G, Diamond, M.P., Saed, G.M. (2015) A single nucleotide polymorphism in catalase is strongly associated with ovarian cancer survival.   PLoS ONE 10(8): e0135739.   Doi: 10.1371/journal.pone.0135739.

Belotte, Jimmy, Nicole M. Fletcher, Awoniyi O. Awonuga, Mitchell Alexis, Husam M. Abu-Soud, Ghassan M. Saed, Michael P. Diamond, and Mohammed G. Saed. "The Role of Oxidative Stress in the Development of Cisplatin Resistance in Epithelial Ovarian Cancer." *Reproductive Sciences* 21, no. 4 (2014): 503–8.

Berek, J.S., Crum, C., and Friedlander, M. (2012). Cancer of the ovary, fallopian tube, and peritoneum. Int J Gynaecol Obstet *119 Suppl 2*, S118-129.

Berge, Wera, Kenneth Mundt, Hung Luu, and Paolo Boffetta. "Genital Use of Talc and Risk of Ovarian Cancer: A Meta-Analysis." *European Journal of Cancer Prevention*, July 2017.

Berry, G., M. L. Newhouse, and J. C. Wagner. "Mortality from All Cancers of Asbestos Factory Workers in East London 1933-80." *Occupational and Environmental Medicine* 57, no. 11 (November 2000): 782–85.

Bertolotti, Marinella, Daniela Ferrante, Dario Mirabelli, Mario Botta, Marinella Nonnato, Annalisa Todesco, Benedetto Terracini, and Corrado Magnani. "[Mortality in the cohort of the asbestos cement workers in the Eternit plant in Casale Monferrato (Italy)]." *Epidemiologia E Prevenzione* 32, no. 4–5 (October 2008): 218–28.

Blount, A.M. (1991). Amphibole content of cosmetic and pharmaceutical talcs. Environ Health Perspect *94*, 225-230.

Blumenkrantz, M. J., N. Gallagher, R. A. Bashore, and H. Tenckhoff. "Retrograde Menstruation in Women Undergoing Chronic Peritoneal Dialysis." *Obstetrics and Gynecology* 57, no. 5 (May 1981): 667–70.

Bodmer, M., Becker, C., Meier, C., Jick, S.S., and Meier, C.R. (2011). Use of metformin and the risk of ovarian cancer: a case-control analysis. Gynecol Oncol *123*, 200-204.

Bonovas, S., Filioussi, K., and Sitaras, N.M. (2005). Do nonsteroidal anti-inflammatory drugs affect the risk of developing ovarian cancer? A meta-analysis. Br J Clin Pharmacol *60*, 194-203.

Booth, M, V Beral, and P Smith. "Risk Factors for Ovarian Cancer: A Case-Control Study." *British Journal of Cancer* 60, No. 4 (October 1989): 592–98.

Boorman, G. A., and J. C. Seely. "The Lack of an Ovarian Effect of Lifetime Talc Exposure in F344/N Rats and B6C3F1 Mice." *Regulatory Toxicology and Pharmacology: RTP* 21, no. 2 (April 1995): 242–43.

Bowtell, D.D. (2010). The genesis and evolution of high-grade serous ovarian cancer. Nat Rev Cancer *10*, 803-808.

Buz'Zard, Amber R., and Benjamin H. S. Lau. "Pycnogenol® Reduces Talc-Induced Neoplastic Transformation in Human Ovarian Cell Cultures." *Phytotherapy Research* 21, No. 6 (June 2007): 579–86.

Camargo, M.C., Stayner, L.T., Straif, K., Reina, M., Al-Alem, U., Demers, P.A., and Landrigan, P.J. (2011). Occupational exposure to asbestos and ovarian cancer: a meta-analysis. Environ Health Perspect *119*, 1211-1217.

Carr, C.J. "Talc: Consumer Uses and Health Perspectives" 21 (1995): 211–15.

Casagrande, J.T., Louie, E.W., Pike, M.C., Roy, S., Ross, R.K., and Henderson, B.E. (1979). "Incessant ovulation" and ovarian cancer. Lancet *2*, 170-173.

Chan, J.K., Cheung, M.K., Husain, A., Teng, N.N., West, D., Whittemore, A.S., Berek, J.S., and Osann, K. (2006). Patterns and progress in ovarian cancer over 14 years. Obstet Gynecol *108*, 521-528.

Chang, Stella, and Harvey A. Risch. "Perineal Talc Exposure and Risk of Ovarian Carcinoma." *Cancer* 79, No. 12 (June 15, 1997): 2396–2401.

Charbonneau, B., Goode, E.L., Kalli, K.R., Knutson, K.L., and Derycke, M.S. (2013). The immune system in the pathogenesis of ovarian cancer. Crit Rev Immunol *33*, 137-164.

Chen Yong. "Risk Factors for Epithelial Ovarian Cancer in Beijing, China." *International Journal of Epidemiology,* 21, No. 1 (1992): 23-29.

Chen, L-M, et al. "Epithelial Carcinoma of the Ovary, Fallopian Tube, and Peritoneum: Epidemiology and Risk Factors - UpToDate," 2018. https://www.uptodate.com/contents/epithelial-carcinoma-of-the-ovary-fallopian-tube-and-periton eum-epidemiology-and-risk-factors?search=Epithelial%20carcinoma%20of%20the%20ovary,% 20fallopian%20tube,%20and%20peritoneum:%20Epidemiology%20and%20risk%20factors&so

urce=search_result&selectedTitle=1~150&usage_type=default&display_rank=1.

Committee on Practice Bulletins–Gynecology, Committee on Genetics, Society of Gynecologic Oncology. "Practice Bulletin No 182: Hereditary Breast and Ovarian Cancer Syndrome." *Obstetrics and Gynecology* 130, no. 3 (2017): e110–26.

Cook, L. S., M. L. Kamb, and N. S. Weiss. "Perineal Powder Exposure and the Risk of Ovarian Cancer." *American Journal of Epidemiology* 145, No. 5 (March 1, 1997): 459–65.

Coussens, L.M., Tinkle, C.L., Hanahan, D., and Werb, Z. (2000). MMP-9 supplied by bone marrow-derived cells contributes to skin carcinogenesis. Cell *103*, 481-490.

Coussens, L.M., and Werb, Z. (2002). Inflammation and cancer. Nature *420*, 860-867.

Cralley, L. J., M. M. Key, D. H. Groth, W. S. Lainhart, and R. M. Ligo. "Fibrous and Mineral Content of Cosmetic Talcum Products." *American Industrial Hygiene Association Journal* 29, no. 4 (August 1968): 350–54.

Cramer, Daniel. "Perineal Talc Exposure and Subsequent Epithelial Ovarian Cancer: A Case-Control Study." *Obstetrics & Gynecology*, (1999).

Cramer, Daniel, et al. "Genital Talc Exposure and Risk of Ovarian Cancer." *Int. J. Cancer:* 81 (1999): 351-356.

Cramer, D. W., W. R. Welch, R. E. Scully, and C. A. Wojciechowski. "Ovarian Cancer and Talc: A Case-Control Study." *Cancer* 50, no. 2 (July 15, 1982): 372–76.

Cramer, Daniel W., Linda Titus-Ernstoff, John R. McKolanis, William R. Welch, Allison F. Vitonis, Ross S. Berkowitz, and Olivera J. Finn. "Conditions Associated with Antibodies Against the Tumor-Associated Antigen MUC1 and Their Relationship to Risk for Ovarian Cancer." *Cancer Epidemiology Biomarkers & Prevention* 14, no. 5 (May 1, 2005): 1125–31.

Cramer, Daniel W., Allison F. Vitonis, Kathryn L. Terry, William R. Welch, and Linda J. Titus. "The Association Between Talc Use and Ovarian Cancer: A Retrospective Case–Control Study in Two US States." *Epidemiology* 27, No. 3 (May 2016): 334–46.

Cramer, Daniel W., William R. Welch, Robert E. Scully, and Carol A. Wojciechowski. "Ovarian Cancer and Talc. A Case-Control Study." *Cancer* 50, No. 2 (July 15, 1982): 372–76.

Crusz, Shanthini M., and Frances R Balkwill. "Inflammation and Cancer: Advances and New Agents." *Nature Reviews Clinical Oncology* 12 (October 2015): 584–96.

Dixon, Suzanne C., Christina M. Nagle, Nicolas Wentzensen, Britton Trabert, Alicia Beeghly-Fadiel, Joellen M. Schildkraut, Kirsten B. Moysich, et al. "Use of Common Analgesic Medications and Ovarian Cancer Survival: Results from a Pooled Analysis in the Ovarian Cancer Association Consortium." *British Journal of Cancer* 116, no. 9 (April 25, 2017):

1223–28.

Dvorak, H.F. (1986). Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. N Engl J Med *315*, 1650-1659.

Egli, G. E., and M. Newton. "The Transport of Carbon Particles in the Human Female Reproductive Tract." *Fertility and Sterility* 12 (April 1961): 151–55.

Eng, Kevin H., J. Brian Szender, John Lewis Etter, Jasmine Kaur, Samantha Poblete, Ruea-Yea Huang, Qianqian Zhu, et al. "Paternal Lineage Early Onset Hereditary Ovarian Cancers: A Familial Ovarian Cancer Registry Study." *PLoS Genetics* 14, no. 2 (February 2018): e1007194.

Fasching, Peter A., Simon Gayther, Leigh Pearce, Joellen M. Schildkraut, Ellen Goode, Falk Thiel, Georgia Chenevix-Trench, et al. "Role of Genetic Polymorphisms and Ovarian Cancer Susceptibility." *Molecular Oncology* 3, No. 2 (April 2009): 171–81.

FDA. "Ltr to Samuel S. Epstein, M.D., RE: Docket Numbers 94P-0420 and FDA-2008-P-0309-0001 /CP," April 1, 2017.

Fernandes, José Veríssimo, Ricardo Ney Oliveira Cobucci, Carlos André Nunes Jatobá, Thales Allyrio Araújo de Medeiros Fernandes, Judson Welber Veríssimo de Azevedo, and Josélio Maria Galvão de Araújo. "The Role of the Mediators of Inflammation in Cancer Development." *Pathology & Oncology Research* 21, no. 3 (July 2015): 527–34.

Ferrante, Daniela, Marinella Bertolotti, Annalisa Todesco, Dario Mirabelli, Benedetto Terracini, and Corrado Magnani. "Cancer Mortality and Incidence of Mesothelioma in a Cohort of Wives of Asbestos Workers in Casale Monferrato, Italy." *Environmental Health Perspectives* 115, no. 10 (October 2007): 1401–5.

Fiume, Monice M., Ivan Boyer, Wilma F. Bergfeld, Donald V. Belsito, Ronald A. Hill, Curtis D. Klaassen, Daniel C. Liebler, et al. "Safety Assessment of Talc as Used in Cosmetics." *International Journal of Toxicology* 34, no. 1 suppl (July 1, 2015): 66S-129S.

Fletcher, N.M., Belotte, J., Saed, M.G., Memaj, I., Diamond, M.P., Morris, R.T., Saed, G.M. Specific point mutations in key redox enzymes are associated with chemoresistance in epithelial ovarian cancer.   Free Radical Biology and Medicine (2017) 102:122-132.

Fletcher, Nicole., Ira Memaj, Ghassan M. Saed. "Talcum Powder Enhances Oxidative Stress in Ovarian Cancer Cells." *Reproductive Sciences*, 25 Supp. 1 (March 2018): 214A-15A.

Fletcher, N.M., Saed, G.M.   (2018). Talcum Powder enhances cancer antigen 125 levels in ovarian cancer cells and in normal ovarian epithelial cells.   LB-044.   Poster presented at: Society for Reproductive Investigation.   65th annual meeting.   San Diego, CA.

Folkins, Ann K. "Chapter 24 - Assessing Pelvic Epithelial Cancer Risk and Intercepting Early Malignancy," *Diagnostic Gynecologic and Obstetric Pathology (Third Edition)*, (2018):

844-864.

Franklin, R.A., and Li, M.O. (2016). Ontogeny of Tumor-associated Macrophages and Its Implication in Cancer Regulation. Trends Cancer *2*, 20-34.

Freedman, Ralph S, Michael Deavers, Jinsong Liu, and Ena Wang. "Peritoneal Inflammation – A Microenvironment for Epithelial Ovarian Cancer (EOC)." *Journal of Translational Medicine* 2, no. 23 (2004).

Friebel, Tara M., Susan M. Domchek, and Timothy R. Rebbeck. "Modifiers of Cancer Risk in BRCA1 and BRCA2 Mutation Carriers: Systematic Review and Meta-Analysis." *Journal of the National Cancer Institute* 106, no. 6 (June 2014): dju091.

Galea, Sandro and Roger D. Vaughan. "Moving Beyond the Cause Constraint: A Public Health of Consequence." *AJPH*, 108, No. 5 (May 2018): 602-603.

Gates, M. A., B. A. Rosner, J. L. Hecht, and S. S. Tworoger. "Risk Factors for Epithelial Ovarian Cancer by Histologic Subtype." *American Journal of Epidemiology* 171, No. 1 (2010): 45–53.

Gates, M. A., S. S. Tworoger, K. L. Terry, L. Titus-Ernstoff, B. Rosner, I. De Vivo, D. W. Cramer, and S. E. Hankinson. "Talc Use, Variants of the GSTM1, GSTT1, and NAT2 Genes, and Risk of Epithelial Ovarian Cancer." *Cancer Epidemiol Biomarkers Prev.* 17, No. 9 (September 2008): 2436–44.

Germani, D., S. Belli, C. Bruno, M. Grignoli, M. Nesti, R. Pirastu, and P. Comba. "Cohort Mortality Study of Women Compensated for Asbestosis in Italy." *American Journal of Industrial Medicine* 36, no. 1 (July 1999): 129–34.

Gertig, Dorota M., David J. Hunter, Daniel W. Cramer, Graham A. Colditz, Frank E. Speizer, Walter C. Willett, and Susan E. Hankinson. "Prospective Study of Talc Use and Ovarian Cancer." *J Natl Cancer Inst* 92, No. 3 (February 2, 2000): 249–52.

Ghio, Andrew J., Joleen M. Soukup, Lisa A. Dailey, Judy H. Richards, Jennifer L. Turi, Elizabeth N. Pavlisko, and Victor L. Roggli. "Disruption of Iron Homeostasis in Mesothelial Cells after Talc Pleurodesis." *American Journal of Respiratory Cell and Molecular Biology* 46, no. 1 (January 1, 2012): 80–86.

Godard, Beatrice, William D. Foulkes, Diane Provencher, Jean-Sebastien Brunet, Patricia N. Tonin, Anne-Marie Mes-Masson, Steven A. Narod, and Parviz Ghadirian. "Risk Factors for Familial and Sporadic Ovarian Cancer among French Canadians: A Case-Control Study." *Am J Obstet Gynecol* 179, No. 2 (August 1998): 403–10.

Gonzalez, Nicole L., Katie M. O'Brien, Aimee A. D'Aloisio, Dale P. Sandler, and Clarice R. Weinberg. "Douching, Talc Use, and Risk of Ovarian Cancer:" *Epidemiology* 27, No. 6 (November 2016): 797–802.

Gordon, Ronald E., Sean Fitzgerald, and James Millette. "Asbestos in Commercial Cosmetic

Talcum Powder as a Cause of Mesothelioma in Women." *International Journal of Occupational and Environmental Health* 20, no. 4 (October 2014): 318–32.

Green, Adèle, David Purdie, Christopher Bain, Victor Siskind, Peter Russell, Michael Quinn, and Bruce Ward. "Tubal Sterilisation, Hysterectomy and Decreased Risk of Ovarian Cancer." *Int. J Cancer* 71, No. 6 (June 11, 1997): 948–51.

Grivennikov, Sergei I., Florian R. Greten, and Michael Karin. "Immunity, Inflammation, and Cancer." *Cell* 140, no. 6 (March 19, 2010): 883–99.

Gross, Alan J. and Paul H. Berg.   "A Meta-Analytical Approach Examining the Potential Relationship Between Talc Exposure and Ovarian Cancer." *Journal of Exposure Analysis and Environmental Epidemiology*, 5, No. 2 (1995): 181-195.

Halme, J., M. G. Hammond, J. F. Hulka, S. G. Raj, and L. M. Talbert. "Retrograde Menstruation in Healthy Women and in Patients with Endometriosis." *Obstetrics and Gynecology* 64, no. 2 (August 1984): 151–54.

Hamilton, T.C., Fox, H., Buckley, C.H., Henderson, W.J., and Griffiths, K.   Effects of talc on the rat ovary.   Br. J. Exp. Path.   (1984) 65: 101-106.

Harlow, Bernard L., and Noel S. Weiss. "A Case-Control Study of Borderline Ovarian Tumors: The Influence of Perineal Exposure to Talc." *American Journal of Epidemiology* 130, No. 2 (August 1989): 390–94.

Harlow, Bernard L., Daniel W. Cramer, Debra A. Bell and William R. Welch. "Perineal Exposure to Talc and Ovarian Cancer Risk." *Obstet Gynecol* 80, No. 1 (1992): 19-26.

Harper, A.K., Saed, G.M.   Talc induces a pro-oxidant state in normal and ovarian cancer cells through gene point mutations in key redox enzymes.   Accepted for presentation at SGO meeting 2019.   [In Press].

Hartge, Patricia, Robert Hoover, Linda Lesher and Larry McGowan.   "Talc and Ovarian Cancer." *JAMA*, 250, No. 14 (1983): 1844.

Hein, M.J., Stayner, L.T., Lehman, E., Dement, J.M.   Follow-up study of chrysotile textile workers: cohort mortality and exposure-response.   Occup Environ Med (2007) 64:616-625.

Heller, D.S., Gordon, R.E., Westhoff, C., and Gerber, S. (1996). Asbestos exposure and ovarian fiber burden. Am J Ind Med *29*, 435-439.

Henderson, W. J., T. C. Hamilton, M. S. Baylis, C. G. Pierrepoint, and K. Griffiths. "The Demonstration of the Migration of Talc from the Vagina and Posterior Uterus to the Ovary in the Rat." *Environmental Research* 40, no. 2 (August 1986): 247–50.

Henderson, W. J., C. A. Joslin, A. C. Turnbull, and K. Griffiths. "Talc and Carcinoma of the Ovary and Cervix." *The Journal of Obstetrics and Gynaecology of the British Commonwealth*

78, no. 3 (March 1971): 266–72.

Hernán, Miguel A. "The C-Word: Scientific Euphemisms Do Not Improve Causal Inference From Observational Data." *American Journal of Public Health* 108, No. 5 (May 2018): 616–19.

Hesterberg, T.W., Butterick, C.J., Oshimura, M., Brody, A.R., and Barrett, J.C. (1986). Role of phagocytosis in Syrian hamster cell transformation and cytogenetic effects induced by asbestos and short and long glass fibers. Cancer Res *46*, 5795-5802.

Hill, Austin Bradford. "The Environment and Disease: Association or Causation?" *Proceedings of the Royal Society of Medicine* 58, no. 5 (May 1965): 295–300.

Hillegass, Jedd M., Arti Shukla, Maximilian B. MacPherson, Jeffrey P. Bond, Chad Steele, and Brooke T. Mossman. "Utilization of Gene Profiling and Proteomics to Determine Mineral Pathogenicity in a Human Mesothelial Cell Line (LP9/TERT-1)." *Journal of Toxicology and Environmental Health. Part A* 73, no. 5 (January 2010): 423–36.

Houghton, S. C., K. W. Reeves, S. E. Hankinson, L. Crawford, D. Lane, J. Wactawski-Wende, C. A. Thomson, J. K. Ockene, and S. R. Sturgeon. "Perineal Powder Use and Risk of Ovarian Cancer." *Journal of the National Cancer Institute* 106, No. 9 (September 10, 2014).

Huncharek, Michael, J.F. Geschwind and Bruce Kupelnick. "Perineal Application of Cosmetic Talc and Risk of Invasive Epithelial Ovarian Cancer: A Meta-analysis of 11,933 Subjects from Sixteen Observational Studies." *Anticancer Research,* 25 (2003): 1955-1960.

Huncharek, Michael, Joshua Muscat, Adedayo Onitilo, and Bruce Kupelnick. "Use of Cosmetic Talc on Contraceptive Diaphragms and Risk of Ovarian Cancer: A Meta-Analysis of Nine Observational Studies:" *European Journal of Cancer Prevention* 16, No. 5 (October 2007): 422–29.

Hunn, Jessica, and Gustavo C. Rodriguez. "Ovarian Cancer: Etiology, Risk Factors, and Epidemiology." *Clinical Obstetrics and Gynecology* 55, No. 1 (March 2012): 3–23.

"Inflammation: A Hidden Path to Breaking the Spell of Ovarian Cancer." *Cell Cycle* 8, no. 19 (2009): 3107–11.

Institute of Medicine (US) Committee on Asbestos: Selected Health Effects. *Asbestos: Selected Cancers*. The National Academies Collection: Reports Funded by National Institutes of Health. Washington (DC): National Academies Press (US), 2006.

International Agency for Research on Cancer (IARC). "IARC Monographs on the Evaluation of Carcinogenic Risks to Humans – IARC : Asbestos," 1977.

International Agency for Research on Cancer (IARC), "Cobalt in Hard Metals and Cobalt Sulfate, Gallium Arsenide, Indium Phosphide and Vanadium Pentoxide," IARC Monograph No. 86 (2006).

International Agency for Research on Cancer (IARC), "Arsenic, Metals, Fibres, and Dusts," IARC Monograph No. 100C (2009).

International Agency for Research on Cancer (IARC), "Overall Evaluations of Carcinogenicity: An Updating of IARC Monographs Volumes 1 to 42", IARC Supplement No. 7 (1987).

International Agency for Research on Cancer (IARC), "Silica and Some Silicates", IARC Monograph No. 42 (1987).

International Agency for Research on Cancer (IARC), "Carbon Black, Titanium Dioxide, and Talc", IARC Monograph No. 93 (2010).

International Agency for Research on Cancer (IARC), "Arsenic, Metals, Fibres, and Dusts", IARC Monograph No. 100C (2012).

International Agency for Research on Cancer (IARC), "Mechanisms of Fibre Carcinogenesis", IARC Scientific Publications, No. 140 (1996).

Irie, H., Banno, K., Yanokura, M., Iida, M., Adachi, M., Nakamura, K., Umene, K., Nogami, Y., Masuda, K., Kobayashi, Y*., et al.* (2016). Metformin: A candidate for the treatment of gynecological tumors based on drug repositioning. Oncol Lett *11*, 1287-1293.

Iturralde, M., and P. F. Venter. "Hysterosalpingo-Radionuclide Scintigraphy (HERS)." *Seminars in Nuclear Medicine* 11, no. 4 (October 1981): 301–14.

Jaurand, M.C. Mechanisms of fiber-induced genotoxicity. Environ Health Perspect (1997) 105(Suppl 5):1073-1084.

Jayson, G.C., Kohn, E.C., Kitchener, H.C., and Ledermann, J.A. (2014). Ovarian cancer. Lancet *384*, 1376-1388.

Jervis, Sarah, Honglin Song, Andrew Lee, Ed Dicks, Jonathan Tyrer, Patricia Harrington, Douglas F. Easton, Ian J. Jacobs, Paul P. D. Pharoah, and Antonis C. Antoniou. "Ovarian Cancer Familial Relative Risks by Tumour Subtypes and by Known Ovarian Cancer Genetic Susceptibility Variants." *Journal of Medical Genetics* 51, no. 2 (February 2014): 108–13.

Jia, D., Nagaoka, Y., Katsumata, M., and Orsulic, S. (2018). Inflammation is a key contributor to ovarian cancer cell seeding. Sci Rep *8*, 12394.

Jones, Richard E., and Kristin H. Lopez. "Human Reproductive Biology - 4th Edition Chapter 9 - Gamete Transport and Fertilization." In *Human Reproductive Biology*, Third., 159–73. San Diego: Academic Press, 2006.

Jordan, S.J., Cushing-Haugen, K.L., Wicklund, K.G., Doherty, J.A., and Rossing, M.A. (2012). Breast-feeding and risk of epithelial ovarian cancer. Cancer Causes Control *23*, 919-927.

Kane, AB, P Boffetta, R Saracci, and JD Wilbourn. "Mechanisms of Fibre Carcinogenesis." IARC, 1996.

Karageorgi, S., M. A. Gates, S. E. Hankinson, and I. De Vivo. "Perineal Use of Talcum Powder and Endometrial Cancer Risk." *Cancer Epidemiology Biomarkers & Prevention* 19, No. 5 (May 1, 2010): 1269–75.

Kauff, Noah D., Nandita Mitra, Mark E. Robson, Karen E. Hurley, Shaokun Chuai, Deborah Goldfrank, Eve Wadsworth, et al. "Risk of Ovarian Cancer in BRCA1 and BRCA2 Mutation-Negative Hereditary Breast Cancer Families." *Journal of the National Cancer Institute* 97, no. 18 (September 21, 2005): 1382–84.

Keskin, N., Teksen, Y.A., Ongun, E.G., Ozay, Y., Saygili, H. Does long-term talc exposure have a carcinogenic effect on the female genital system of rats? An experimental pilot study. Arch Gynecol Obstet. (2009).   280:925-931.

Kisielewski, R., Tolwinska, A., Mazurek, A., and Laudanski, P. (2013). Inflammation and ovarian cancer--current views. Ginekol Pol *84*, 293-297.

Kissler, Stefan, Ernst Siebzehnruebl, Joachim Kohl, Anja Mueller, Nadja Hamscho, Regine Gaetje, Andre Ahr, Achim Rody, and Manfred Kaufmann. "Uterine Contractility and Directed Sperm Transport Assessed by Hysterosalpingoscintigraphy (HSSG) and Intrauterine Pressure (IUP) Measurement." *Acta Obstetricia Et Gynecologica Scandinavica* 83, no. 4 (April 2004): 369–74.

Khan, Mohd Imran, AmoghA. Sahasrabuddhe, Govil Patil, Mohd Javed Akhtar, Mohd Ashquin, and Iqbal Ahmad. "Nano-Talc Stabilizes TNF- α m-RNA in Human Macrophages." *Journal of Biomedical Nanotechnology* 7, no. 1 (January 1, 2011): 112–13.

Kiraly, Orsolya, Guanyu Gong, Werner Olipitz, Sureshkumar Muthupalani, and Bevin P. Engelward. "Inflammation-Induced Cell Proliferation Potentiates DNA Damage-Induced Mutations In Vivo." *PLoS Genetics*, February 3, 2015.

Kunz, G., D. Beil, H. Deiniger, A. Einspanier, G. Mall, and G. Leyendecker. "The Uterine Peristaltic Pump. Normal and Impeded Sperm Transport within the Female Genital Tract." *Advances in Experimental Medicine and Biology* 424 (1997): 267–77.

Kurta, M. L., K. B. Moysich, J. L. Weissfeld, A. O. Youk, C. H. Bunker, R. P. Edwards, F. Modugno, R. B. Ness, and B. Diergaarde. "Use of Fertility Drugs and Risk of Ovarian Cancer: Results from a U.S.-Based Case-Control Study." *Cancer Epidemiol Biomarkers   Prev.* 21, No. 8 (August 1, 2012): 1282–92.

Landen, Charles N., Michael J. Birrer, and Anil K. Sood. "Early Events in the Pathogenesis of Epithelial Ovarian Cancer." *Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology* 26, no. 6 (February 20, 2008): 995–1005.

Langseth, H., Johansen, B.V., Nesland, J.M., and Kjaerheim, K. (2007). Asbestos fibers in ovarian tissue from Norwegian pulp and paper workers. Int J Gynecol Cancer *17*, 44-49.

Langseth, H, S E Hankinson, J Siemiatycki, and E Weiderpass. "Perineal Use of Talc and Risk of Ovarian Cancer." *J Epidemiol Community Health* 62, No. 4 (April 1, 2008): 358–60.

Langseth, Hilde, and Kristina Kjærheim. "Ovarian Cancer and Occupational Exposure among Pulp and Paper Employees in Norway." *Scand J Work, Environ Health* 30, No. 5 (October 2004): 356–61.

Lee, Jennifer S., Esther M. John, Valerie McGuire, Anna Felberg, Kimberly L. Ostrow, Richard A. DiCioccio, Frederick P. Li, Alexander Miron, Dee W. West, and Alice S. Whittemore. "Breast and Ovarian Cancer in Relatives of Cancer Patients, with and without BRCA Mutations." *Cancer Epidemiology, Biomarkers & Prevention: A Publication of the American Association for Cancer Research, Cosponsored by the American Society of Preventive Oncology* 15, no. 2 (February 2006): 359–63.

Levanon, K., Crum, C., Drapkin, R. New insights into the pathogenesis of serous ovarian cancer and its clinical impact. J Clin Oncol. (2008). Nov 10;26(32):5284-93.

Li, D. (2011). Metformin as an antitumor agent in cancer prevention and treatment. J Diabetes *3*, 320-327.

Lin, H.W., Tu, Y.Y., Lin, S.Y., Su, W.J., Lin, W.L., Lin, W.Z., Wu, S.C., and Lai, Y.L. (2011). Risk of ovarian cancer in women with pelvic inflammatory disease: a population-based study. Lancet Oncol *12*, 900-904.

Liou, Geou-Yarh, and Peter Storz. "Reactive Oxygen Species in Cancer." *Free Radical Research* 44, no. 5 (May 2010): 476–96.

Liu, Y., and Cao, X. (2015). The origin and function of tumor-associated macrophages. Cell Mol Immunol *12*, 1-4.

Lockey, J. E. "Nonasbestos Fibrous Minerals." *Clinics in Chest Medicine* 2, no. 2 (May 1981): 203–18.

"Longo - Feb 2018 MAS Report," 2018.

Longo, William E., and Mark W. Rigler. "The Analysis of Johnson & Johnson's Historical Baby Powder & Shower to Shower Products from the 1960's to the Early 1990's for Amphibole Asbestos," November 14, 2018.

Longo, William E., Mark W. Rigler, and William B. Egeland. "Below the Waist Application of Johnson & Johnson Baby Powder." Materials Analytical Service, LLC, September 2017.

Maccio, A., and Madeddu, C. (2012). Inflammation and ovarian cancer. Cytokine *58*, 133-147.

Mabuchi, S., Kawase, C., Altomare, D.A., Morishige, K., Sawada, K., Hayashi, M., Tsujimoto, M., Yamoto, M., Klein-Szanto, A.J., Schilder, R.J*., et al.* (2009). mTOR is a promising therapeutic target both in cisplatin-sensitive and cisplatin-resistant clear cell carcinoma of the ovary. Clin Cancer Res *15*, 5404-5413.

Magnani, C., D. Ferrante, F. Barone-Adesi, M. Bertolotti, A. Todesco, D. Mirabelli, and B. Terracini. "Cancer Risk after Cessation of Asbestos Exposure: A Cohort Study of Italian Asbestos Cement Workers." *Occupational and Environmental Medicine* 65, no. 3 (March 2008): 164–70.

Mahdavi, A., Pejovic, T., and Nezhat, F. (2006). Induction of ovulation and ovarian cancer: a critical review of the literature. Fertil Steril *85*, 819-826.

Mäki-Nevala, Satu, Virinder Kaur Sarhadi, Aija Knuuttila, Ilari Scheinin, Pekka Ellonen, Sonja Lagström, Mikko Rönty, et al. "Driver Gene and Novel Mutations in Asbestos-Exposed Lung Adenocarcinoma and Malignant Mesothelioma Detected   by Exome Sequencing." *Lung* 194, no. 1 (February 2016): 125–35.

Mallen, Adrianne R., Mary K. Townsend, and Shelley S. Tworoger. "Risk Factors for Ovarian Carcinoma." *Hematol Oncol Clin N Am*, September 2018. https://doi.org/10.1016/j.hoc.2018.07.002.

Mantovani, A., Bottazzi, B., Colotta, F., Sozzani, S., and Ruco, L. (1992a). The origin and function of tumor-associated macrophages. Immunol Today *13*, 265-270.

Mantovani, A., Bussolino, F., and Dejana, E. (1992b). Cytokine regulation of endothelial cell function. FASEB J *6*, 2591-2599.

Mantovani, A., Bussolino, F., and Introna, M. (1997). Cytokine regulation of endothelial cell function: from molecular level to the bedside. Immunol Today *18*, 231-240.

Melaiu, Ombretta, Federica Gemignani, and Stefano Landi. "The Genetic Susceptibility in the Development of Malignant Pleural Mesothelioma." *Journal of Thoracic Disease* 10, no. Suppl 2 (January 2018): S246–52.

Meng, Qingsong, Weixue Sun, John Jiang, Nicole M. Fletcher, Michael P. Diamond, and Ghassan M. Saed. "Identification of Common Mechanisms between Endometriosis and Ovarian Cancer." *Journal of Assisted Reproduction and Genetics* 28 (2011): 917–23.

Merritt, Melissa A., Adèle C. Green, Christina M. Nagle, Penelope M. Webb, and Australian Cancer Study (Ovarian Cancer) and Australian Ovarian Cancer Study Group. "Talcum Powder, Chronic Pelvic Inflammation and NSAIDs in Relation to Risk of Epithelial Ovarian Cancer." *Int. J. Cancer* 122, No. 1 (2008): 170–76.

Merritt, M.A., Green, A.C., Nagle, C.M., Webb, P.M., Australian Cancer Study (Ovarian Cancer) and Australian Ovarian Cancer Study Group. Talcum powder, chronic pelvic inflammation and NSAIDs in relation to risk of epithelial ovarian cancer. Int. J. Cancer. (2008)

122:170-176.

Mills, P.K., Riordan, D.G., Cress, R.D., and Young, H.A. (2004). Perineal talc exposure and epithelial ovarian cancer risk in the Central Valley of California. Int J Cancer *112*, 458-464.

Milne, R. L., and A. C. Antoniou. "Genetic Modifiers of Cancer Risk for BRCA1 and BRCA2 Mutation Carriers." *Annals of Oncology: Official Journal of the European Society for Medical Oncology* 22 Suppl 1 (January 2011): i11-17.

Milne, Roger L., and Antonis C. Antoniou. "Modifiers of Breast and Ovarian Cancer Risks for BRCA1 and BRCA2 Mutation Carriers." *Endocrine-Related Cancer* 23, no. 10 (2016): T69-84. https://doi.org/10.1530/ERC-16-0277.

Modan, B., Hartge, P., Hirsh-Yechezkel, G., Chetrit, A., Lubin, F., Beller, U., Ben-Baruch, G., Fishman, A., Menczer, J., Struewing, J.P.*, et al.* (2001). Parity, oral contraceptives, and the risk of ovarian cancer among carriers and noncarriers of a BRCA1 or BRCA2 mutation. N Engl J Med *345*, 235-240.

Moon, Min Chaul, Jung Duck Park, Byung Soon Choi, So Young Park, Dong Won Kim, Yong Hyun Chung, Naomi Hisanaga, and Il Je Yu. "Risk Assessment of Baby Powder Exposure through Inhalation." *Toxicological Research* 27, no. 3 (September 2011): 137–41.

Moorman, P. G., R. T. Palmieri, L. Akushevich, A. Berchuck, and J. M. Schildkraut. "Ovarian Cancer Risk Factors in African-American and White Women." *Am J Epidemiol* 170, No. 5 (September 1, 2009): 598–606.

Mor, G., Yin, G., Chefetz, I., Yang, Y., and Alvero, A. (2011). Ovarian cancer stem cells and inflammation. Cancer Biol Ther *11*, 708-713.

Mostafa, S. A., C. B. Bargeron, R. W. Flower, N. B. Rosenshein, T. H. Parmley, and J. D. Woodruff. "Foreign Body Granulomas in Normal Ovaries." *Obstetrics and Gynecology* 66, no. 5 (November 1985): 701–2.

Muscat, J. E., and M. S. Huncharek. "Causation and Disease: Biomedical Science in Toxic Tort Litigation." *Journal of Occupational Medicine.: Official Publication of the Industrial Medical Association* 31, no. 12 (December 1989): 997–1002.

Nadler, Diana L., and Igor G. Zurbenko. "Estimating Cancer Latency Times Using a Weibull Model," 2014, 8.

Narod, S.A., Risch, H., Moslehi, R., Dorum, A., Neuhausen, S., Olsson, H., Provencher, D., Radice, P., Evans, G., Bishop, S.*, et al.* (1998). Oral contraceptives and the risk of hereditary ovarian cancer. Hereditary Ovarian Cancer Clinical Study Group. N Engl J Med *339*, 424-428.

National Toxicology Program. Toxicology and carcinogenesis studies of talc (CAS No. 14807-96-6) in F344/N rats and B6C3F$_1$ mice (Inhalation studies); 1993. Report No. NTP TR 421; NIH Publication No. 93-3152.

Nelson, Heather H., and Karl T. Kelsey. "The Molecular Epidemiology of Asbestos and Tobacco in Lung Cancer." *Oncogene* 21, no. 48 (October 21, 2002): 7284–88.

Ness, R. B., and C. Cottreau. "Possible Role of Ovarian Epithelial Inflammation in Ovarian Cancer." *JNCI Journal of the National Cancer Institute* 91, no. 17 (September 1, 1999): 1459–67. https://doi.org/10.1093/jnci/91.17.1459.

Ness, Roberta B., Jeane Ann Grisso, Carrie Cottreau, Jennifer Klapper, Ron Vergona, James E. Wheeler, Mark Morgan, and James J. Schlesselman. "Factors Related to Inflammation of the Ovarian Epithelium and Risk of Ovarian Cancer." *Epidemiology* 11, No. 2 (2000): 111–17.

Newhouse, M. L., G. Berry, J. C. Wagner, and M. E. Turok. "A Study of the Mortality of Female Asbestos Workers." *British Journal of Industrial Medicine* 29, no. 2 (April 1972): 134–41.

NIOSH - CDC – Occupational Cancer – Carcinogen List, May 2012

"NIOSH 2011 Current Intelligence Bulletin No. 62," 2011.

NIOSH. "Asbestos Fibers and Other Elongated Mineral Particles: State of the Science and Roadmap for Research (Revised Draft)," January 2009.

NIOSH. "Fiber Exposure during Use of Baby Powders, Report No. IWS-36-6.," July 1972..

"NTP Toxicology and Carcinogenesis Studies of Talc (CAS No. 14807-96-6)(NonAsbestiform) in F344/N.Rats and B6C3Fl Mice (Inhalation Studies)," 1993.

Nunes, S.C., and Serpa, J. (2018). Glutathione in Ovarian Cancer: A Double-Edged Sword. Int J Mol Sci *19*.

Oberdörster, Günter, Eva Oberdörster, and Jan Oberdörster. "Nanotoxicology: An Emerging Discipline Evolving from Studies of Ultrafine Particles." *Environmental Health Perspectives* 113, no. 7 (July 2005): 823–39.

Paoletti, L., Caiazza, S., Donelli, G., and Pocchiari, F. (1984). Evaluation by electron microscopy techniques of asbestos contamination in industrial, cosmetic, and pharmaceutical talcs. Regul Toxicol Pharmacol *4*, 222-235.

Paluch-Shimon, S., Cardoso, F., Sessa, C., Balmana, J., Cardoso, M.J., Gilbert, F., Senkus, E., and Committee, E.G. (2016). Prevention and screening in BRCA mutation carriers and other breast/ovarian hereditary cancer syndromes: ESMO Clinical Practice Guidelines for cancer prevention and screening. Ann Oncol *27*, v103-v110.

Paoletti, L., Caiazza, S., Donelli, G., and Pocchiari, F. (1984). Evaluation by electron microscopy techniques of asbestos contamination in industrial, cosmetic, and pharmaceutical talcs. Regul Toxicol Pharmacol *4*, 222-235.

Pardoll, D.M. (2002). Spinning molecular immunology into successful immunotherapy. Nat Rev Immunol *2*, 227-238.

Park, Hyo K., Joellen M. Schildkraut, Anthony J. Alberg, Elisa V. Bandera, Jill S. Barnholtz-Sloan, Melissa Bondy, Sydnee Crankshaw, et al. "Benign Gynecologic Conditions Are Associated with Ovarian Cancer Risk in African-American Women: A Case–Control Study." *Cancer Causes & Control*, September 29, 2018.

Parmley, T. H., and J. D. Woodruff. "The Ovarian Mesothelioma." *American Journal of Obstetrics and Gynecology* 120, no. 2 (September 15, 1974): 234–41

Peshkin, B., and et al. "Genetic Counseling and Testing for Hereditary Breast and Ovarian Cancer - UpToDate," 2018.
https://www.uptodate.com/contents/genetic-counseling-and-testing-for-hereditary-breast-and-ova rian-cancer?search=Genetic%20counseling%20and%20testing%20for%20hereditary%20breast %20and%20ovarian%20cancer&source=search_result&selectedTitle=1~150&usage_type=defau lt&display_rank=1.
———. "Overview of Hereditary Breast and Ovarian Cancer Syndromes - UpToDate," 2018.
https://www.uptodate.com/contents/overview-of-hereditary-breast-and-ovarian-cancer-syndrome s?search=Overview%20of%20hereditary%20breast%20and%20ovarian%20cancer%20syndrome s&source=search_result&selectedTitle=1~150&usage_type=default&display_rank=1.
———. "Prevalence of BRCA1 and BRCA2 Mutations and Associated Cancer Risks - UpToDate," 2018.
https://www.uptodate.com/contents/prevalence-of-brca1-and-brca2-mutations-and-associated-ca ncer-risks?search=prevalence-of-brca1-and-brca2-mu%E2%80%A6search_result%26selectedTit le%3D1~73%26usage_type%3Ddefault%26display_rank%3D1&source=search_result&selected Title=2~150&usage_type=default&display_rank=2.

Pejovic, T., and Nezhat, F. (2011). Missing link: inflammation and ovarian cancer. Lancet Oncol *12*, 833-834.

Penninkilampi, Ross and Guy D. Eslick. "Perineal Talc Use and Ovarian Cancer: A Systematic Review and Meta-Analysis." *Epidemiology* 29, No. 1 (January 2018): 41-49.

Phillips, J. C., P. J. Young, K. Hardy, and S. D. Gangolli. "Studies on the Absorption and Disposition of 3H-Labelled Talc in the Rat, Mouse, Guinea-Pig and Rabbit." *Food and Cosmetics Toxicology* 16, no. 2 (April 1978): 161–63.

Pinto, Mauricio, Carlos Balmaceda, Maria L. Bravo, Sumie Kato, Alejandra Villarroel, et al. "Patient Inflammatory Status and CD4+/CD8+ Intraepithelial Tumor Lymphocyte Infiltration are Predictors of Outcomes in High-Grade Serous Ovarian Cancer." *Gynecologic Oncology* (2018).

Pira, E, C Pelucchi, L Buffoni, A Palmas, M Turbiglio, E Negri, P G Piolatto, and C La Vecchia. "Cancer Mortality in a Cohort of Asbestos Textile Workers." *British Journal of Cancer* 92, no. 3 (February 2005): 580–86. https://doi.org/10.1038/sj.bjc.6602240.

Pira, Enrico, Canzio Romano, Francesco S. Violante, Andrea Farioli, Giovanna Spatari, Carlo La

Vecchia, and Paolo Boffetta. "Updated Mortality Study of a Cohort of Asbestos Textile Workers." *Cancer Medicine* 5, no. 9 (2016): 2623–28.

Pukkala, Eero, Jan Ivar Martinsen, Elsebeth Lynge, Holmfridur Kolbrun Gunnarsdottir, Pär Sparén, Laufey Tryggvadottir, Elisabete Weiderpass, and Kristina Kjaerheim. "Occupation and Cancer - Follow-up of 15 Million People in Five Nordic Countries." *Acta Oncologica (Stockholm, Sweden)* 48, no. 5 (2009): 646–790.

Purdie, David, Adèle Green, Christopher Bain, Victor Siskind, Bruce Ward, Neville Hacker, Michael Quinn, Gordon Wright, Peter Russell, and Beatrice Susil. "Reproductive and Other Factors and Risk of Epithelial Ovarian Cancer: An Australian Case-Control Study." *International Journal of Cancer* 62, No. 6 (September 15, 1995): 678–84.

Radic, I, I Vucak, J Milosevic, A Marusic, S Vukicevic, and M Marusic. "Immunosuppression Induced by Talc Granulomatosis in the Rat." *Clinical and Experimental Immunology* 73, no. 2 (August 1988): 316–21.

Ramus, S.J., Vierkant, R.A., Johnatty, S.E., Pike, M.C., Van Den Berg, D.J., Wu, A.H., Pearce, C.L., Menon, U., Gentry-Maharaj, A., Gayther, S.A*., et al.* (2008). Consortium analysis of 7 candidate SNPs for ovarian cancer. Int J Cancer *123*, 380-388.

Rebbeck, T.R., Mitra, N, Wan, F., Sinilnikova, O.M., Healey, S., McGuffog, L., Mazoyer, S., Chenevix-Trench, G., Easton, D.F., Antoniou, A.C., Nathanson, K.L., the CIMBA Consortium. Association fo type and location of BRCA1 and BRCA2 mutations with risk of breast and ovarian cancer. JAMA. (2015).   April 7; 313(13):1347-1361.

Reid, B.M., Permuth, J.B., Sellers, T.A. Epidemiology of ovarian cancer: a review. Cancer Med Biol. (February 2017) Vol 14, No 1.

Reid, A., J. Heyworth, N. de Klerk, and A. W. Musk. "The Mortality of Women Exposed Environmentally and Domestically to Blue Asbestos at Wittenoom, Western Australia." *Occupational and Environmental Medicine* 65, no. 11 (November 2008): 743–49. https://doi.org/10.1136/oem.2007.035782.

Reid, A., N. de Klerk, and A. W. Musk. "Does Exposure to Asbestos Cause Ovarian Cancer? A Systematic Literature Review and Meta-Analysis." *Cancer Epidemiology Biomarkers & Prevention* 20, no. 7 (July 1, 2011): 1287–95.

Reid, Alison, Amanda Segal, Jane S. Heyworth, Nicholas H. de Klerk, and Arthur W. Musk. "Gynecologic and Breast Cancers in Women after Exposure to Blue Asbestos at Wittenoom." *Cancer Epidemiology, Biomarkers & Prevention: A Publication of the American Association for Cancer Research, Cosponsored by the American Society of Preventive Oncology* 18, no. 1 (January 2009): 140–47.

Reuter, Simone, Subash C. Gupta, Madan M. Chaturvedi, and Bharat B. Aggarwal. "Oxidative Stress, Inflammation, and Cancer: How Are They Linked?" *Free Radical Biology and Medicine* 49, no. 11 (December 1, 2010): 1603–16.

Ring, Kari L., Christine Garcia, Martha H. Thomas, and Susan C. Modesitt. "Current and Future Role of Genetic Screening in Gynecologic Malignancies." *American Journal of Obstetrics and Gynecology* 217, no. 5 (2017): 512–21.

Rohl, A. N. "Asbestos in Talc." *Environmental Health Perspectives* 9 (December 1974): 129–32.

Rohl, A. N., A. M. Langer, I. J. Selikoff, A. Tordini, R. Klimentidis, D. R. Bowes, and D. L. Skinner. "Consumer Talcums and Powders: Mineral and Chemical Characterization." *Journal of Toxicology and Environmental Health* 2, no. 2 (November 1976): 255–84.

Rosenblatt, Karin A., Moyses Szklo, and Neil B. Rosenshein. "Mineral Fiber Exposure and the Development of Ovarian Cancer." *Gynecologic Oncology* 45, No. 1 (April 1992): 20–25.

Rosenblatt, Karin A., Noel S. Weiss, Kara L. Cushing-Haugen, Kristine G. Wicklund, and Mary Anne Rossing. "Genital Powder Exposure and the Risk of Epithelial Ovarian Cancer." *Cancer Causes Control* 22, No. 5 (May 2011): 737–42.

Rösler, J. A., H. J. Woitowitz, H. J. Lange, R. H. Woitowitz, K. Ulm, and K. Rödelsperger. "Mortality Rates in a Female Cohort Following Asbestos Exposure in Germany." *Journal of Occupational Medicine.: Official Publication of the Industrial Medical Association* 36, no. 8 (August 1994): 889–93.

Ross, M. "Geology, Asbestos, and Health." *Environmental Health Perspectives* 9 (December 1974): 123–24.

Saed, Ghassan M., Rouba Ali-Fehmi, Zhong L. Jiang, Nicole M. Fletcher, Michael P. Diamond, Husam M. Abu-Soud, and Adnan R. Munkarah. "Myeloperoxidase Serves as a Redox Switch That Regulates Apoptosis in Epithelial Ovarian Cancer." *Gynecologic Oncology* 116, no. 2 (February 2010): 276–81. https://doi.org/10.1016/j.ygyno.2009.11.004.

Saed, G.M., Diamond, M.P., and Fletcher, N.M. (2017). Updates of the role of oxidative stress in the pathogenesis of ovarian cancer. Gynecol Oncol *145*, 595-602.

Saed, G.M., Morris, R.T., and Fletcher, N.M. (2018). New Insights into the Pathogenesis of Ovarian Cancer: Oxidative Stress. In Ovarian Cancer-From Pathogenesis to Treatment (IntechOpen).

Savant, Sudha S., Shruthi Sriramkumar and Heather M. O'Hagan. "The Role of Inflammation and Inflammatory Mediators in the Development, Progression, Metastasis, and Chemoresistance of Epithelial Ovarian Cancer." *Cancers* 10, No. 251 (2018).

Schildkraut, J. M., S. E. Abbott, A. J. Alberg, E. V. Bandera, J. S. Barnholtz-Sloan, M. L. Bondy, M. L. Cote, et al. "Association between Body Powder Use and Ovarian Cancer: The African American Cancer Epidemiology Study (AACES)." *Cancer Epidemiol Biomarkers Prev.* 25, No. 10 (October 1, 2016): 1411–17.

Sellers, T.A., Huang, Y., Cunningham, J., Goode, E.L., Sutphen, R., Vierkant, R.A., Kelemen, L.E., Fredericksen, Z.S., Liebow, M., Pankratz, V.S.*, et al.* (2008). Association of single nucleotide polymorphisms in glycosylation genes with risk of epithelial ovarian cancer. Cancer Epidemiol Biomarkers Prev *17*, 397-404.

Shan, W., and Liu, J. (2009). Inflammation: a hidden path to breaking the spell of ovarian cancer. Cell Cycle *8*, 3107-3111.

Shen, H., Fridley, B.L., Song, H., Lawrenson, K., Cunningham, J.M., Ramus, S.J., Cicek, M.S., Tyrer, J., Stram, D., Larson, M.C.*, et al.* (2013). Epigenetic analysis leads to identification of HNF1B as a subtype-specific susceptibility gene for ovarian cancer. Nat Commun *4*, 1628.

Sellers, T.A., Huang, Y., Cunningham, J., Goode, E.L., Sutphen, R., Vierkant, R.A., Kelemen, L.E., Fredericksen, Z.S., Liebow, M., Pankratz, V.S.*, et al.* (2008). Association of single nucleotide polymorphisms in glycosylation genes with risk of epithelial ovarian cancer. Cancer Epidemiol Biomarkers Prev *17*, 397-404.

Shukla, A., MacPherson, M.B., Hillegass, J., Ramos-Nino, M.E., Alexeeva, V., Vacek, P.M., Bond, J.P., Pass, H.I., Steele, C., Mossman, B.T. Alterations in gene expression in human mesothelial cells correlate with mineral pathogenicity. Am J Respir Cell Mol Biol. (2009). 41:114-123.

Shushan, Asher, Orn Pnlticl, and JoRe Iscovich. "Human Menopausal Gonadotropin and the Risk of Epithelial Ovarian Cancer." *Gynecol-Endocrin* 65, No. 1 (January 1996).

Siegel, R.L., Miller, K.D., and Jemal, A. (2015). Cancer statistics, 2015. CA Cancer J Clin *65*, 5-29.

Sjösten, A. C. E., H. Ellis, and G. a. B. Edelstam. "Retrograde Migration of Glove Powder in the Human Female Genital Tract." *Human Reproduction* 19, no. 4 (April 1, 2004): 991–95.

Soong, Thing Rinda, Brooke E. Howitt, Alexander Miron, Neil S. Horowitz, Frank Campbell, Colleen M. Feltmate, Michael G. Muto, et al. "Evidence for Lineage Continuity between Early Serous Proliferations (ESPs) in the Fallopian Tube and Disseminated High-Grade Serous Carcinomas." *The Journal of Pathology*, July 25, 2018.

Starita, Lea M., Muhtadi M. Islam, Tapahsama Banerjee, Aleksandra I. Adamovich, Justin Gullingsrud, et al. "A Multiplex Homology-Directed DNA Repair Assay Reveals the Impact of More Than 1,000 BRCA1 Missense Substitution Variants on Protein Function." *American Journal of Human Genetics* 103, (October 4, 2018): 1-11.

Steiling, W., J. F. Almeida, H. Assaf Vandecasteele, S. Gilpin, T. Kawamoto, L. O'Keeffe, G. Pappa, K. Rettinger, H. Rothe, and A. M. Bowden. "Principles for the Safety Evaluation of Cosmetic Powders." *Toxicology Letters*, August 17, 2018.

Steiling, W., M. Bascompta, P. Carthew, G. Catalano, N. Corea, A. D'Haese, P. Jackson, et al. "Principle Considerations for the Risk Assessment of Sprayed Consumer Products." *Toxicology*

Letters 227, no. 1 (May 16, 2014): 41–49.

Stewart, Louise M., Katrina Spilsbury, Susan Jordan, Colin Stewart, C. D'Arcy J. Holman, Aime Powell, Joanne Reekie, and Paul Cohen. "Risk of High-Grade Serous Ovarian Cancer Associated with Pelvic Inflammatory Disease, Parity and Breast Cancer." *Cancer Epidemiology* 55 (August 2018): 110–16.

Straif, Kurt, Lamia Benbrahim-Tallaa, Robert Baan, Yann Grosse, Béatrice Secretan, Fatiha El Ghissassi, Véronique Bouvard, et al. "A Review of Human Carcinogens—Part C: Metals, Arsenic, Dusts, and Fibres." *The Lancet Oncology* 10, No. 5 (May 2009): 453–54.

Terry, K. L., S. Karageorgi, Y. B. Shvetsov, M. A. Merritt, G. Lurie, P. J. Thompson, M. E. Carney, et al. "Genital Powder Use and Risk of Ovarian Cancer: A Pooled Analysis of 8,525 Cases and 9,859 Controls." *Cancer Prev Res* 6, No. 8 (August 2013): 811–21.

Titus-Ernstoff, L., Perez, K., Cramer, D.W., Harlow, B.L., Baron, J.A., and Greenberg, E.R. (2001). Menstrual and reproductive factors in relation to ovarian cancer risk. Br J Cancer *84*, 714-721.

Torre, L.A., Bray, F., Siegel, R.L., Ferlay, J., Lortet-Tieulent, J., and Jemal, A. (2015). Global cancer statistics, 2012. CA Cancer J Clin *65*, 87-108.

Trabert, B., R. B. Ness, W.-H. Lo-Ciganic, M. A. Murphy, E. L. Goode, E. M. Poole, L. A. Brinton, et al. "Aspirin, Nonaspirin Nonsteroidal Anti-Inflammatory Drug, and Acetaminophen Use and Risk of Invasive Epithelial Ovarian Cancer: A Pooled Analysis in the Ovarian Cancer Association Consortium." *J Natl Cancer Inst* 106, No. 2 (February 6, 2014).

Trabert, Britton, Elizabeth M Poole, Emily White, Kala Visvanathan, Hans-Olov Adami, Garnet L Anderson, Theodore M Brasky, et al. "Analgesic Use and Ovarian Cancer Risk: An Analysis in the Ovarian Cancer Cohort Consortium." *J Natl Cancer Inst*, 111, No. 2 (May 31, 2018).

Tzonou, Anastasia, Argy Polychronopoulou, Chung-cheng Hsieh, Apostolos Rebelakos, Anna Karakatsani and Dimitrios Trichopoulos. "Hair Dyes, Analgesics, Tranquilizers and Perineal Talc Application as Risk Factors for Ovarian Cancer," *Int. J. Cancer*, 55, (1993): 408-410.

U.S. Environmental Protection Agency (USEPA), "Arsenic, inorganic", in the Integrated Risk Information System database listing (1995). Available at the USEPA Website: https://cfpub.epa.gov/ncea/iris/iris_documents/documents/subst/0278_summary

US EPA. "Health Assessment Document for Talc. | National Technical Reports Library - NTIS." -600/8-91/217, 1992. https://ntrl.ntis.gov/NTRL/dashboard/searchResults/titleDetail/PB92239524.xhtml.

Vallyathan N.V. and J.E. Craighead, "Pulmonary pathology in workers exposed to nonasbestiform talc", Human Pathology 12(1):28-35 (1981).

Van Gosen, B. S., H.A. Lowers, S.J. Sutley, and C.A. Gent. "Using the Geologic Setting of Talc

Deposits as an Indicator of Amphibole Asbestos Content." *Environmental Geology* 45, no. 7 (2004): 20.

Vasama-Neuvonen, K., E. Pukkala, H. Paakkulainen, P. Mutanen, E. Weiderpass, P. Boffetta, N. Shen, T. Kauppinen, H. Vainio, and T. Partanen. "Ovarian Cancer and Occupational Exposures in Finland." *American Journal of Industrial Medicine* 36, no. 1 (July 1999): 83–89.

Venkatesan, Priya. "Possible X Chromosome-Linked Transmission of Ovarian Cancer." *The Lancet. Oncology* 19, no. 4 (April 2018): e185.

Venter, P. F., and M. Iturralde. "Migration of a Particulate Radioactive Tracer from the Vagina to the Peritoneal Cavity and Ovaries." *South African Medical Journal = Suid-Afrikaanse Tydskrif Vir Geneeskunde* 55, no. 23 (June 2, 1979): 917–19.

Verdoodt, Freija, Christian Dehlendorff, Søren Friis, and Susanne K. Kjaer. "Non-Aspirin NSAID Use and Ovarian Cancer Mortality." *Gynecologic Oncology* 150, no. 2 (2018): 331–37.

Virta, RL. "The Phase Relationship of Talc and Amphiboles in a Fibrous Talc Sample." IH; Report of Investigations, 1985.

Vitonis, A.F., Titus-Ernstoff, L., and Cramer, D.W. (2011). Assessing ovarian cancer risk when considering elective oophorectomy at the time of hysterectomy. Obstet Gynecol *117*, 1042-1050.

Wang, N.S., Jaurand, M.C., Magne, L., Kheuang, L., Pinchon, M.C., and Bignon, J. (1987). The interactions between asbestos fibers and metaphase chromosomes of rat pleural mesothelial cells in culture. A scanning and transmission electron microscopic study. Am J Pathol *126*, 343-349.

Wang, Q. (2016) "Cancer predisposition genes: molecular mechanisms and clinical impact on personalized cancer care: examples of Lynch and HBOC syndromes."Acta Pharmacologica Sinica 37:143-149.

Wehner, A.P. (1994). Biological effects of cosmetic talc. Food Chem Toxicol *32*, 1173-1184.

Wehner, A. P., A. S. Hall, R. E. Weller, E. A. Lepel, and R. E. Schirmer. "Do Particles Translocate from the Vagina to the Oviducts and Beyond?" *Food and Chemical Toxicology: An International Journal Published for the British Industrial Biological Research Association* 23, no. 3 (March 1985): 367–72.

Wehner, A. P., R. E. Weller, and E. A. Lepel. "On Talc Translocation from the Vagina to the Oviducts and Beyond." *Food and Chemical Toxicology: An International Journal Published for the British Industrial Biological Research Association* 24, no. 4 (April 1986): 329–38.

Wendel, Jillian R. Hufgard, Xiyin Wang and Shannon M. Hawkins. "The Endometriotic Tumor Microenvironment in Ovarian Cancer." *Cancers* 10, No. 261 (2018).

Werner, I. "Presence of Asbestos in Talc Samples." *Atemschutzinform* 21, no. 5 (1982).

Whittemore, Alice S, Marion L Wu, Ralphs Paffenbarger, L Sarles, James B Kampert, and S'Tella Grosser. "Personal and Environmental Characteristics Related to Epithelial Ovarian Cancer," *Am J Epidemiol*, 128, No. 6 (1988) 1228-1240.

Wignall, B.K., and A.J. Fox. "Mortality of Female Gas Mask Assemblers." *British Journal of Industrial Medicine* 39, no. 1 (1982): 34–38.

Wong, C. "Perineal Talc Exposure and Subsequent Epithelial Ovarian Cancer: A Case-Control Study." *Obstetrics & Gynecology* 93, No. 3 (March 1999): 372–76.

Woodruff, J. D. "The Pathogenesis of Ovarian Neoplasia." *The Johns Hopkins Medical Journal* 144, no. 4 (April 1979): 117–20.

Wu, A.H., Pearce, C.L., Tseng, C.C., Templeman, C., and Pike, M.C. (2009). Markers of inflammation and risk of ovarian cancer in Los Angeles County. Int J Cancer *124*, 1409-1415.

Wu, A. H., C. L. Pearce, C.-C. Tseng, and M. C. Pike. "African Americans and Hispanics Remain at Lower Risk of Ovarian Cancer Than Non-Hispanic Whites after Considering Nongenetic Risk Factors and Oophorectomy Rates." *Cancer Epidemiol Biomarkers Prev* 24, No. 7 (July 2015): 1094–1100.

Yegles, M., Saint-Etienne, L., Renier, A., Janson, X., and Jaurand, M.C. (1993). Induction of metaphase and anaphase/telophase abnormalities by asbestos fibers in rat pleural mesothelial cells in vitro. Am J Respir Cell Mol Biol *9*, 186-191.

Yilmaz, Ercan, Mehmet Gul, Rauf Melekoglu, Isil Koleli. "Immunhistochemical Analysis of Nuclear Factor Kappa Beta Expression in Etiopathogenesis of Ovarian Tumors." *Acta Cir Bras.* 33, No. 7 (2018): 641-650.

Zazenski, R., W. H. Ashton, D. Briggs, M. Chudkowski, J. W. Kelse, L. MacEachern, E. F. McCarthy, M. A. Nordhauser, M. T. Roddy, and N. M. Teetsel. "Talc: Occurrence, Characterization, and Consumer Applications." *Regulatory Toxicology and Pharmacology: RTP* 21, no. 2 (April 1995): 218–29.

Zervomanoklakis, I, H.W. Ott, D Hadziomerovic, V. Mattle, B.E. Seeber, I. Virgolini, D. Heute, S. Kissler, G. Leyendecker, and L. Wildt. "Physiology of Upward Transport in the Human Female Genital Tract." *Annals of New York Acadamy of Sciences* 1101, no. 1 (2007): 1–20.

Depositions

Deposition of Alice M. Blount in Gail Lucille Ingham, et al. v. Johnson & Johnson, et al.

Depositions of John Hopkins (Aug 16 and 17, 2018; Oct 26, 2018; Nov 5, 2018)

Deposition of Julie Pier (Sept. 12 and 13, 2018)

Expert Reports

Expert Report of Michael Crowley, PhD (Nov. 15, 2018)

Expert Report of William E. Longo, PhD and Mark W. Rigler PhD (Nov. 14, 2018)

Expert Report of William E. Longo, PhD and Mark W. Rigler PhD. Analysis of J&J Baby Powder & Valiant Shower to Shower Talc Products for Amphibole (Tremolite) Asbestos Expert Report.   August 2, 2017.

Expert Report of William E. Longo, PhD and Mark W. Rigler PhD.   TEM Analysis of Historical 1978 Johnson's Baby Powder Sample for Amphibole Asbestos .   February 16, 2018.

Documents Produced

JNJ 000018679-90
JNJTALC000864509-732

Exhibit 74

Chapter 2

Molecular Analysis of Genetic Instability Caused by Chronic Inflammation

Bin Yan, Yuanlin Peng, and Chuan-Yuan Li

Summary

Genetic instability is a hallmark of human cancers. It is the driving force for tumor development as it facilitates the accumulation of mutations in genes that regulate cell death and proliferation and therefore promotes malignant transformation. Chronic inflammation is a common underlying condition for human tumor development, accounting for approximately 20% of human cancers. TNFα is an important inflammation cytokine and is crucial to the development of inflammation-associated cancers. We have shown that TNFα can cause DNA damages through reactive oxygen species (ROS). TNFα treatment in cultured cells resulted in increased gene mutations, gene amplification, micronuclei formation and chromosomal instability. Antioxidants significantly reduced TNFα-induced genetic damage. In addition, TNFα treatment alone led to increased malignant transformation of mouse embryo fibroblasts, which could be partially suppressed by antioxidants. Therefore, genetic instability plays an important role in inflammation-associated cancers.

Key words: Genetic instability; Inflammation; Cancer; Reactive oxygen species; 8-Oxo-deoxyguanosine.

1. Introduction

1.1. Inflammation and Cancer

Exposure to environmental carcinogen and chronic inflammation are two important underlying conditions for sporadic human tumor development. Chronic inflammations predispose patients to cancers. For example, chronic atrophic gastritis increases the risk for gastric cancer, chronic hepatitis for hepatic carcinoma and chronic skin ulcer for squamous cancer of the skin.

Most studies on the mechanism underlying inflammation-associated cancers focused on NFκB signaling. We recently found that ROS-induced DNA damage and genetic instability is another

16   Yan, Peng, and Li

important contributing factor in the development of cancers in chronic inflammation *(1)*. ROS is abundant in inflammation. It can be produced by the respiratory burst in the inflammatory cells or induced by inflammatory cytokines such as TNFα. We have found that TNFα induces ROS, causes genetic aberrations and leads to transformation, all of which were at least partially inhibited by antioxidants *(1)*.

1.2. Genetic Assays   In order to study instability in inflammation-associated cancers, a variety of genetic assays can be applied to assess the oxidative stress and DNA damages, which include micronucleus assay, cytogenetic analysis of chromosomal aberrations, gene amplification assay, mutation assay, comet assay and immunostaining for 8-oxodG and γ-H2AX. The malignant transformation resulting from accumulation of mutations can be determined by transformation assays such as soft agar assay. This chapter will elaborate the detailed protocols for these assays.

2. Materials

2.1. Cell Culture

1. 379.2 cells are $p53^{-/-}$ colon carcinoma HCT116 cells, which were kindly provided by Dr. Bert Vogelstein of Johns Hopkins University, Baltimore, MD. 379.2 cells were cultured in McCoy 5A medium supplemented with 10% fetal bovine serum. L929 cells were maintained in DMEM medium supplemented with 10% equine serum. 10T1/2 and BALB/3T3 are mouse embryonic fibroblasts obtained from Cell Culture Facility of Duke University Comprehensive Cancer Center (Durham, NC). They were maintained in DMEM medium supplemented with 10% fetal bovine serum.

2. Recombinant human and mouse TNFα were purchased from R&D Systems, Inc (Minneapolis, MN 55413). *N*-acetyl cystein (NAC), Vitamin C, Vitamin E, actinomycin D, rotenone and dichlorofluorescein diacetate (DCFDA) were purchased from Sigma (St. Louis, MO).

2.2. Gene Amplification Assay

1. The selective agent for *cad* gene amplification, PALA, was obtained from the Drug Synthesis Branch, Division of Cancer treatment, National Cancer Institute.

2. Methotrexate (MTX) was purchased from Sigma (St. Louis, MO).

3. Methylene blue solution: 0.4% methylene blue dissolved in 30% methanol solution.

4. 1% Acetic acid solution: 1% acetate in 50% methanol.

5. Crystal violet (CV) staining fixative: 0.5% CV in 80% methanol.

6. Dissolves PALA/MTX in ddH$_2$O to make 1,000× stock solution.

2.3. Analysis of Chromosomal Aberrations

2.3.1. In Vitro Analysis of Chromosomal Aberrations in Cultured Cells

1. Colcemid (10 μg/ml) is purchased from Gibco (now Invitrogen) or Ivine Scientific.

2. 0.5% Colchicine (Sigma C3915) in water (see recipe; store at –20°C).

3. 1-cc disposable syringes and 23 gauge needles.

4. 15-ml Conical centrifuge tubes (Corning #25310).

5. Hypotonic solution: 0.075 M KCl (0.56% KCl) in ddH$_2$O.

6. Fixative: 3 volume of methanol mixed with 1 volume of acetic acid.

7. Giemsa stain solution: (Dissolving one tablet in 100 ml ddH$_2$O and add 5 ml R66 solution. Both the tablet and R66 solution were purchased from BDH Laboratory Supplies (Poole, England)).

2.3.2. Fluorescence In Situ Hybridization (Suitable for Chromosome Painting)

1. Hybridization mixture: mix 2 ml 20× SSC at pH 5.8 and 10 ml formamide, add 2 g dextran sulfate on the top and vortex to mix. Then leave on bench top over night for dextran sulfate to dissolve. Aliquot and store in –20°C.

2. 20× SCC at pH 5.8 and pH 7.4: Mix thoroughly 175.3 g NaCl, 88.2 g sodium citrate in 800 ml ddH$_2$O. Adjust pH, adjust volume to 1 L, store at room temperature for up to 6 months.

3. 70% Formamide denaturation solution: 5 ml 20× SCC at pH 5.8 and 35 ml formamide in 10 ml ddH$_2$O. Store covered between uses. Discard after 2 months.

4. 50% Formamide in 2× SSC: 5 ml 20× SCC at pH 5.8 and 25 ml formamide in 20 ml ddH$_2$O. Store covered between uses. Discard after 2 months.

5. 2× SSC pH 7.4: 5 ml 20× SCC at pH 7.4 in 45 ml ddH$_2$O. Discard after use.

6. PN buffer: Use 0.1 M NaH$_2$PO$_4$ to adjust pH of the 0.1 M Na$_2$HPO$_4$ to achieve pH 8.0 and add 0.05% Nonidet® P-40. Autoclave and store up to 6 months.

7. For chromosome painting, whole chromosome probes for mouse chromosomes were purchased from Cambio Ltd, Cambridge, UK.

2.4. Detection of Aneuploid Cells by FACS Analysis

1. Wash buffer: PBS + 1% fetal bovine serum (FBS).

2. Fixative: cold 70% ethanol.

3. Staining buffer: propidium iodide (10 μg/ml) and ribonuclease A (100 μg/ml) in PBS.

18   Yan, Peng, and Li

4. Flow cytometry machine and ModFit LT cell-cycle analysis software (Verity Software House, Topsham, ME).

2.5. Micronucleus Assay

1. Cytochalasin B (Sigma, St. Louis, MO, USA) stock solution: 5 mg/ml dissolved in ethanol.

2. Carnoy fixative (ratio of volume: methanol:acetic acid = 3:1).

3. 2×SSC buffer with NP40: 2× sodium chloride–sodium citrate (SSC) buffer with 0.1% Nonidet® P 40 (NP 40).

4. Acridine orange (Sigma-Aldrich) staining buffer: 5–10 µg/ml acridine orange (AO) dissolved in 0.1 M sodium phosphate (pH 7.2) + equal volume of heptane.

5. Fluorescence microscope with FITC filter.

2.6. Immunodetection for 8-oxodG in Mouse Tissues

1. Fixative: acetone: Methanol (1:1).

2. M.O.M.™ Immunodetection Kit FLUORESCEIN (Catalog No. FMK-2201) is purchased from Vector Laboratories, Inc (Burlingame, CA 94010).

3. M.O.M.™ Mouse Ig Blocking Reagent: add two drops of stock solution to 2.5 ml of PBS.

4. M.O.M.™ Diluent: add 600 µl of protein concentrate stock solution to 7.5 ml of PBS.

5. M.O.M.™ Biotinylated Anti-Mouse IgG Reagent: add 10 µl of stock solution to 2.5 ml of M.O.M.™ diluent prepared above.

6. VECTOR ABC Reagent: add two drops of Reagent A to 2.5 ml PBS, mix and then add two drops of Reagent B and mix. Allow ABC Reagent to stand for 30 min prior to use.

7. DAB Enhancing Solution (cat# H-2200) or VECTOR VIP Substrate Kit (cat# SK-4600).

2.7. Soft Agar Assay and Tumorigenesis Assay

1. 10× agar stock: Boil 5% (w/v) bacto-agar (DIFCO, Detroit, MI) stock in distilled water, autoclave to sterilize, and store in 50-ml aliquots at room temperature.

2. Cell culture medium as appropriate for each cell line.

3. 6- to 8-week-old athymic nude mice were purchased from Charles River Laboratories, Raleigh, NC.

3. Methods

3.1. Gene Amplification Assays

Standard gene amplification measures the frequency of amplification of *cad* or *dhfr* gene. *cad* gene encodes carbamyl phosphate synthetase, aspartate transcarbamylase and dihyroorotase. *dhfr*

gene encodes dihydrofolate reductase. The sole known mechanism for PALA resistance is amplification of *cad* gene, therefore frequency of *cad* amplification can be quantified by clonogenic assay with PALA selection. Similarly, MTX can be used to quantify the frequency of *dhfr* gene amplification *(2)*.

1. 50% Lethal dosage (LD_{50}) of the drug has to be determined to decide the concentration of PALA or MTX for each cell line. (1) Seed 2–10×10^3 cells/well depending on the cell size (refer **Subheading 4**) into a 24-well plate; (2) Cells are grown in the presence of a concentration titration of PALA/MTX until the well of cells grow to confluence in the absence of PALA/MTX; (3) Wash cells once with PBS, then stain cells with methylene blue solution for 20 min; (4) Wash cells with distilled water 3–4 times then extract methylene blue with 1 ml 1% acetic acid solution; (5) Measure the absorbance OD650 at $\lambda = 650$ nm then make a graph to determine LD_{50}, the concentration that inhibited 50% cell growth *(3, 4)*.

2. For estimation of resistant clones, about 1–2×10^6 cells were seeded into each P10 dish (10-cm dish). Cells were then selected in 3.5–$9 \times LD_{50}$ of PALA or MTX. Medium should be changed with fresh PALA/MTX every 3–5 days (*see* **Notes 1** and **2**).

3. At the same time when seeding the cells, seed 200–500 cells into at least three plates and let the cells grow in the absence of selective drugs for calculation of plating efficiency (PE). PE = number of colonies formed/number of cells seeded (*see* **Note 3**).

4. Resistant colonies usually appear in 2–3 weeks. Cells were fixed and stained with 0.5% crystal violet in 80% methanol. Colonies were counted subsequently (*see* **Note 4**).

5. Frequency of amplification is expressed as the number of resistant colonies relative to the number of colonies formed without PALA and MTX *(2, 5)*.

3.2. Analysis of Chromosomal Aberrations

3.2.1. In Vitro Analysis of Chromosomal Aberrations in Cultured Cells

1. Colcemid® is added to the cell culture at a final concentration of 0.02 μg/ml. Harvest the cells by 5 min centrifuge at 400 × *g* 1–4 h later depending on how rapidly the cells grow (*see* **Note 5**).

2. Cells are resuspended in 10 ml 0.075 M KCl and incubated at 37°C water bath for 10–15 min (*see* **Note 6**).

3. 2 ml Fixative is added and mixed well at the end of incubation.

4. Cells are spun down and the supernatant is removed. Cells are resuspended in 10 ml fixative at room temperature.

5. **Step 4** is repeated once.

20   Yan, Peng, and Li

6. Cells are then collected by 5 min centrifuge at $400 \times g$ and suspended in 0.5–2 ml fresh fixatives depending on the number of cells (addition of fixative should be just enough to make a thin cell suspension, solution in tube will look slightly opaque) (*see* **Note 7**). Cell suspension is then dropped onto cold wet slides. Cell concentration and cell spreading are monitored by phase contrast microscopy (*see* **Note 8**).

7. Slides are stained with 0.5% Wright stain or Giemsa stain for 10 min. Metaphase spreads are examined under oil immersion microscope for chromosomal aberrations including chromosome breaks, rings, dicentrics, terminal deletions and interstitious deletions (double minutes). The number of chromosome in each spread can be counted under a microscope to assess the abnormality in ploidy.

3.2.2. In Vivo Analysis of Chromosomal Aberrations in Mouse Bone Marrow Cells

This protocol was modified from the one published on The Jackson Laboratory website *(6)*.

1. Inject mouse with 0.1 cc of 0.5% colchicine (stock solution) intraperitoneally. Wait 30–60 min depending on the age of the mice, shorter for young mice and longer for old ones.

2. Sacrifice mouse and remove femur(s) and tibia(s).
Early metaphases seem to be more prevalent in tibias.

3. Cut off just enough of the bone heads to insert a 23 gauge needle into the marrow cavity.

4. Flush out cells into a conical centrifuge tube using a 1-cc syringe filled with 0.075 M KCl.

5. Incubate the tubes at 37°C for 10–15 min.

6. Centrifuge at $400 \times g$ for 5 min in a clinical bench-top centrifuge.

7. Remove supernatant and add 0.5 ml of fixative without disturbing the pellet. Remove fixative after 3–4 s and add 2 ml fresh fixative without disturbing the pellet.

8. Allow tubes to sit at room temperature 30 min.
The procedure can be interrupted at this point and resumed later. Always refrigerate cells if they are to be left standing in fixative longer than 30 min

9. After 30 min centrifuge the cells at $400 \times g$, remove the fixative, and resuspend the cells in fresh fixative.

10. Repeat **step 9** once.

11. Continue as described in **steps 6** and 7 in **Subheading 3.2.1**.

3.2.3. Fluorescence In Situ Hybridization (Suitable for Chromosome Painting)

This protocol was modified from that published by Lichter et al. *(7)* and can be used for chromosome painting to study chromosome translocations.

Pretreatment of Chromosome Slides

1. Apply 20 μl of 100 μg/ml RNase to the target region of the slides at 37°C for 3 min.

2. Wash two times for 3 min in 2× SSC.

3. Place slides in 0.01 N HCl containing 100 mg/L pepsin at 37°C for 5 min (*see* **Notes 9–11**).

4. Wash two times for 5 min in PBS.

5. Dehydrate the slides by placing in 70, 90, and 100% ethanol for 1 min each at room temperature.

6. Air-dry the slides and proceed to slide denaturation. Slides may be used immediately or store at –20°C.

Denaturation of Chromosome Slides

1. Denature the slides for 2 min in 70% formamide denaturation solution at 70°C. For old slides, denature extra 6 s for every 1 month of slides stored.

2. Immediately transfer slides to ice-cold 70% ethanol for 2 min to reduce strand reannealing before adding the probe.

3. Dehydrate slides by placing in 70, 90, and 100% ethanol for 2 min each at room temperature.

4. Allow slides to air-dry for a few minutes.

5. Prewarm slides to 37°C in an incubator.

Probe Denaturation and Hybridization

1. Mix thoroughly 0.5 μl fluorochrome labeled probe (~100 ng), 9 μl Hybridization mixture, 2 μl of Cot-1 DNA (~2 μg), and 3.5 μl water.

2. Denature the probe by incubating at 82°C for 10 min (this may be done in a PCR machine for convenience) (*see* **Note 12**).

3. Prehybridization of the probe mixture with excess unlabeled genomic or Cot-1 DNA (usually at least 20 times the amount of the probe DNA) for 30 min to 1 h at 37°C is necessary to reduce the diffuse hybridization of repetitive sequences in the probe to multiple chromosome sites.

4. Spin briefly to collect probe cocktail.

5. Apply the 15 μl denatured and prehybridized probe cocktail onto the denatured chromosome slide and overlay with a 22 × 22 mm coverslip.

6. Seal the coverslip with rubber cement to prevent evaporation during hybridization.

7. Place slides in a pre-warmed dark box and incubate 16–18 h at 37°C. Depending on the concentration and complexity of the probe, hybridization time may vary from overnight to several days without detrimental effects (*see* **Note 13**).

22   Yan, Peng, and Li

Posthybridization Washing Steps

1. Carefully remove rubber cement. Do not remove the coverslips.
2. Place slides 4 min each in two jars containing prewarmed 50% formamide in 2× SSC at 42°C. The coverslips will come off (see **Note 14**).
3. Place slide 4 min each in two jars containing prewarmed 2× SSC at 42°C.
4. Place slides 4 min each in two jars containing prewarmed PN buffer at 42°C.

Detection

1. Do not allow the slides to dry after the washing steps.
2. Apply 15 µl DAPI counterstain and antifade solution to the target area of the slides and apply coverslip.
3. Perform microscopic analysis.

See **Fig. 1** for an example of the FISH.
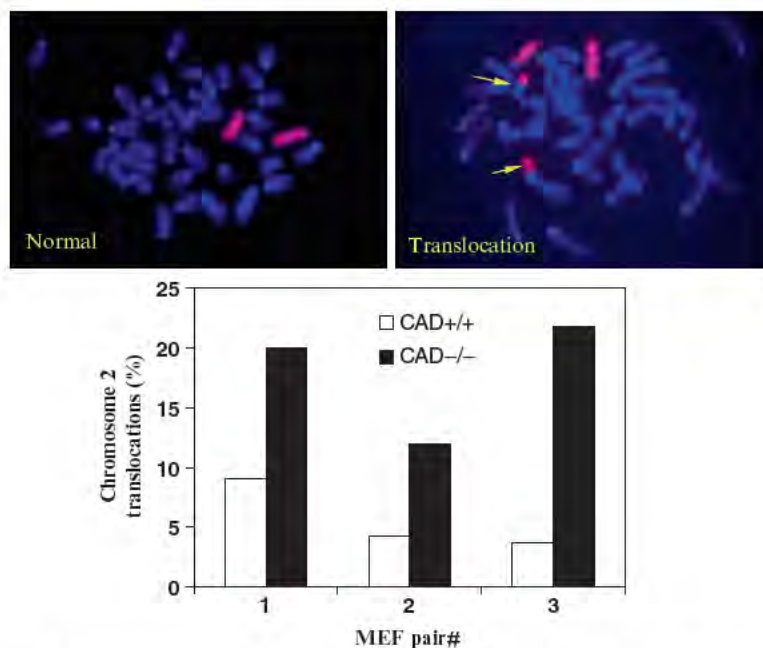
Fig. 1. Whole chromosome painting by FISH. MEF cells from CAD(+/+) and CAD(−/−) cells were irradiated with 4 Gy of γ-rays and then cultured for 12 days. Metaphase chromosome spreads were then prepared. Subsequently, chromosome painting was carried out for the chromosome spreads by use of fluorescently labeled whole chromosome 2 probes. The *top two panels* shows the fluorescent photomicrographs of typical spreads with normal (*left*) and translocated (*right*) chromosome 2. *Arrows* show the chromosomes that are involved in translocations. The *lower panel* shows elevated frequency of radiation-induced chromosome 2 translocations in embryonic fibroblast cells derived from CAD(−/−) mice. Results from three independent pairs of CAD(+/+) and CAD(−/−) mouse embryonic fibroblasts. For each condition, at least 100 metaphases were counted for each experimental point.

3.3. Detection of Aneuploid Cells by FACS Analysis

1. Cells were collected, washed with PBS + 1% FBS (*see* **Note 15**).

2. Cells are centrifuged and supernatant removed. Cold (4°C) 70% ethanol is added to the cell pellet drop by drop with gentle mixing to resuspend the cells. Fix the cells in cold ethanol for at least 3–6 h. At this point, cells can be left in the refrigerator for a couple of weeks.

3. Cells are centrifuged and stained with propidium iodide (10 μg/ml) and ribonuclease A (100 μg/ml) for at least 1 h but no more than 24–48 h.

4. Cells are then subjected to cell cycle analysis using FACS. A percentage of aneuploid cells was calculated with ModFit LT cell-cycle analysis software *(8)* (*see* **Notes 16–18**).

3.4. Micronucleus Assay

1. Cells were plated in the 6-well plates at about 20–30% confluence 24 h before treatment.

2. Treat cells with irradiation, TNFα or other genotoxic agents.

3. After treatment, cytochalasin B was added to the medium at the final concentration of 5 μg/ml and cultured for 48 h.

4. Then the medium was removed and the cells were rinsed with PBS and fixed by Carnoy fixative for 5 min. Then the cells were dried in the air.

5. Then the cells were immersed in the 2× SSC buffer with 0.1% NP 40 for 1 min.

6. After drying in the air, the cells were stained in acridine orange (AO) staining buffer for 2–5 min by gently shaking them (*see* **Note 19**).

7. Then the cells were washed with PBS and the micronuclei were scored under a fluorescent microscope. The criteria for identifying micronucleus is elaborated by Michael Fenech *(9)* (*see* **Notes 20–21**).

3.5. Immunodetection for 8-oxodG in Mouse Tissues

8-oxo-Deoxyguanosine (8-oxodG) is one of the major DNA lesions formed upon oxidative attack of DNA. It is an indicator of oxidative stress as well as a mutagenic adduct that has been associated with pathological states such as cancer and aging *(10)*. The only available oxodG antibody is mouse monoclonal. In order to decrease the background staining due to the presence of endogenous mouse immunoglobins in the mouse tissue, the M.O.M.™ immunodetection kit is used for the immunostaining. Below it is modified from the instruction of the kit.

1. The frozen tissue sections are fixed in ice-cold fixative (acetone:methanol = 1:1) for 10 min and then the slides are air-dried.

2. Wash sections in PBS for 4 min × 3 times.

24   Yan, Peng, and Li

3. Incubate sections with 0.3% hydrogen peroxide in 0.3% normal horse serum in PBS for 10 min to quench endogenous peroxidase activity.

4. Wash section 3 min × 3 in PBS.

5. Incubate sections for 1–4 h in working solution of M.O.M. mouse Ig blocking reagent.

6. Wash slides for about 3 × 3 min in PBS.

7. Incubate slides for 5 min in working solution of M.O.M. diluents (*see* **Note 22**).

8. Dilute 8-oxodG antibody in M.O.M. diluents to 1:1,000. Incubate section in diluted primary antibody for 30–60 min at room temperature or overnight at 4°C.

9. Wash slides for about 3 × 3 min in PBS.

10. Apply working solution of M.O.M. biotinylated anti-mouse IgG reagent and incubate sections for 10 min (*see* **Note 23**).

11. Wash slides for about 3 × 3 min in PBS.

12. Apply VECTASTAIN ABC reagent and incubate the sections for 5 min.

13. Wash slides for about 3 × 5 min in PBS.

14. Prepare and apply peroxidase substrate solution according to substrate kit instructions and develop for 2–10 min with DAB or 2–15 min with VECTOR VIP (*see* **Notes 24–25**).

See **Fig. 2** for an example of the staining.

3.6. Soft Agar Assay for Cellular Transformation

One of the best in vitro indicators of a potential malignant growth is the ability of cells to grow in an anchorage-independent manner. Growth in semi-solid agar media is the most common assay *(11)*.

1. Prepare the 0.5% agar bottom layer by mixing 1 volume of 5% agar (melted by microwave and cooled in 45°C water bath) with 9 volume of medium prewarmed to 45°C. Pipette 5 ml of the 0.5% agar/medium into each 60-mm dish and allow to solidify at room temperature (*see* **Note 26**).

2. Trypsinize the cells and prepare a serial dilutions of single cell suspension to 5×10^4, 2×10^4, 10^4, 5×10^3, 2×10^3, 5×10^2, and 2×10^2 cells/ml.

3. Briefly warm 1 ml of each dilution of cell suspension to 40°C and mix it with 2 ml of the warm 0.5% agar/medium to a final concentration of 0.33% agar in a 15-ml tube, then transfer the cells to the hardened 0.5% agar base layer (*see* **Note 27**).

4. Incubate cells at 37°C in a humidified 5–10% CO_2 environment. Feed cells twice a week by dropwise addition of the growth medium. Score for the presence and frequency of colonies after 2–3 weeks (*see* **Note 28**).

Molecular Analysis of Genetic Instability Caused by Chronic Inflammation   25
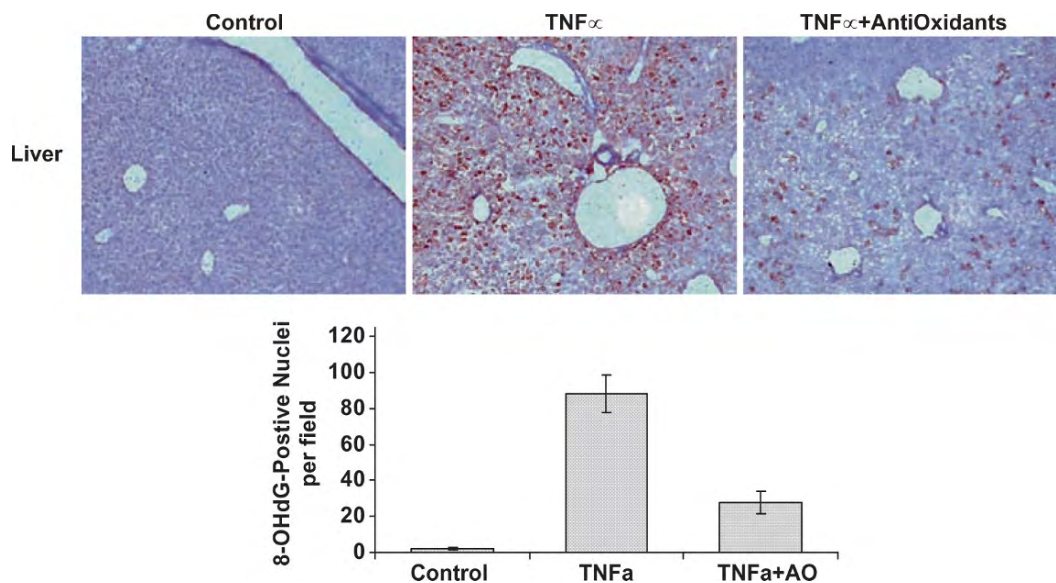
Fig. 2. Induction of 8-OHdG by TNFα in mouse liver tissue. Expression of TNFα in mice was achieved by hydrodynamic delivery of N1-TNFα expression plasmid. Four micrograms of DNA in a calculated volume were injected into each mouse over a period of 5 s. Mice were sacrificed 7 h after injection and tissues were removed and frozen in liquid nitrogen and subsequently stained with an antibody specific to 8-OHdG. The staining of liver tissue is shown in the *upper panel*. Quantification of the 8-OHdG-positive cells is in the *lower panel.*

5. To confirm the tumorigenicity of the colonies that emerged in the soft agar assay, the colonies were picked, transferred into DMEM medium and expanded. Cells from each colony (about $3–5 \times 10^6$) were then injected subcutaneously into the right flanks of 6- to 8-week-old athymic nude mice. After inoculation, the incidence and growth of tumors were evaluated at least once a week for at least 16 weeks. Mice were sacrificed/dissected when tumor size reached 15 mm in diameter (*see* **Note 29**).

4. Notes

1. When seeding cells to each well of a 12-well plate to determine the LD_{50}, seed more cells (up to 10^4) if the cells are small (such as HCT116), less cells ($2–5 \times 10^3$) if the cells are big such as fibroblasts.

2. To compare cad gene amplification between different cell lines, the concentration of PALA/MTX for each cell line is determined by its LD_{50}. Use the same times of LD_{50} instead of the same concentration for each cell line.

26   Yan, Peng, and Li

3. Be very gentle when changing the medium, add the medium to the side of dishes in order not to flush the cell clumps and disperse them.

4. Only colonies with more than 50 cells are counted.

5. For slow growing cells such as fibroblasts, only the floating cells are collected for hypotonic incubation and fixation, every 3–5 h. Combine 2–3 harvests together. The cells in metaphase are round and not attached to the plate. Therefore we can concentrate these cells in this way.

6. Incubation in the hypotonic solution should not exceed 15 min otherwise the chromosome will be fussy; When resuspending the cells after spinning them down, suck out all but 200 µl supernatant and resuspend the cells in this 200 leftover, then resuspend them to the final large volume.

7. Cells can be stored at 4°C for a couple of days at **step 4**. Cells can be stored in methanol at –20°C for longer time if necessary.

8. Dropping cell suspension onto slides is done in a blowing hood to accelerate drying for good spreading. Slides should be tilted and the height to drop is about 20–30 cm. Slides should be cleaned by detergent and water followed by ethanol and dried in the air before use.

9. Careful chromosome slide preparation is the first essential step of a successful hybridization. Usually prepare slides 1 day prior to hybridization. For a long-term storage, keep slides in a desiccator at –20°C. To use freshly made slides, incubate slides in 90°C oven for 10 min, followed by a 60-min incubation in 2× SSC at 37°C and dehydration in 70, 90, and 100% ethanol for 2 min each.

10. Slide pretreatment procedure may not be necessary if it is very clean. Enzymatic treatments reduce the background by digesting RNA and change the accessibility of the chromosome DNA by removing the cytoplasmic proteins.

11. For experiment of one slide, place the Coplin jar containing 70% formamide denaturation solution in the 70°C water bath approximately 30 min prior to use to bring the denaturation solution to 70°C. For every extra slide, increase the temperature setting 0.5°C to maintain the denaturation temperature at 70°C. Immerse no more than four slides in the solution simultaneously.

12. Start the probe denaturation during pretreatment and denaturation of chromosome slides. Time the procedure so that it is completed approximately the same time as the slide denaturation.

13. To prevent photo bleaching, handle all reagents and slides containing fluorochromes in reduced light.

14. Place the six Coplin jars containing two jars each of the 50% formamide in 2× SSC, 2× SSC, and PN buffer in the 42°C water bath approximately 30 min prior to use to bring the washing solutions to 42°C.

15. A normal diploid control should be set for each analysis. The best normal control is peripheral blood lymphocytes or bone marrow cells.

16. When comparing different samples, in order for the analysis to be accurate, equal amount of PI staining buffer should be added to stain equal amount of cells of different samples.

17. Cells should be analyzed within 24 h after staining for the best result.

18. The analysis should be performed by an experienced flow cytometry expert using the ModFit LT cell-cycle analysis software. Since researchers are generally not familiar with this software, it is important to have someone with experience to analyze the data.

19. The AO solution mixed with hepatan form two layers. Take the lower portion containing AO to stain the cells.

20. If the nuclei look fussy under microscope when dry, 0.5 ml PBS can be added to the well and the image will become clear.

21. For the criteria of identifying MN or other nuclear abnormalities reflecting DNA damages such as the bridge reference can be seen in ?, *see (9)*.

22. There are two tricks in decreasing the background caused by endogenous mouse Ig: (1) Permeabilize the tissue with 0.2% Triton X100 in PBS for 20 min at 4°C so that the endogenous Ig can be washed away in the following wash steps; (2) Use acetone instead of paraformaldehyde for fixation because paraformaldehyde can cross link proteins and make it hard to wash the endogenous Ig away.

23. Not all background present in a tissue section will be caused by endogenous mouse IgG. Appropriate negative control sections should be run in parallel, to rule out other possible causes of background.

24. Development times may differ depending upon the level of antigen, the intensity of the stain that is required or the substrate used.

25. During the staining procedure, do not allow the section to dry out. If necessary, use a humidified chamber for incubations.

26. The 0.5% agar/medium and the melted 5% agar should be kept in 45°C water baths during the experiment. They quickly solidify at room temperature.

27. In **step 3**, mixing the cell suspension with the 0.5% agar/medium should be done promptly and thoroughly before it solidifies.

28   Yan, Peng, and Li

28. It usually takes about 3 weeks for the colonies to form. Those with >50 cells are counted as transformed colonies.

29. The limitations of this assay are reflected in the observations that some normal cells do grow in suspension, and that many human tumor cells fail to grow in suspension *(11)*. Therefore, the transformation property of cells forming soft agar colonies should be confirmed by tumorigenesis assay by inoculating cells into nude mice and observing tumor formation. Focus-formation assay can also be used to test cells that do not form colonies in soft agar medium.

References

1. Yan, B., Wang, H., Rabbani, Z.N., Zhao, Y., Li, W., Yuan, Y., Li, F., Dewhirst, M.W., and Li, C.Y. (2006) Tumor necrosis factor-alpha is a potent endogenous mutagen that promotes cellular transformation. *Cancer Res* **66**, 11565–11570.

2. Yan, B., Wang, H., Peng, Y., et al. (2006) A unique role of the DNA fragmentation factor in maintaining genomic stability. *Proc Natl Acad Sci U S A* **103**, 1504–1509.

3. Yin, Y., Tainsky, M.A., Bischoff, F.Z., Strong, L.C., and Wahl, G.M. (1992) Wild-type p53 restores cell cycle control and inhibits gene amplification in cells with mutant p53 alleles. *Cell* **70**, 937–948.

4. Livingstone, L.R., White, A., Sprouse, J., Livanos, E., Jacks, T., and Tlsty, T.D. (1992) Altered cell cycle arrest and gene amplification potential accompany loss of wild-type p53. *Cell* **70**, 923–935.

5. Chen, S., Bigner, S.H., and Modrich, P. (2001) High rate of CAD gene amplification in human cells deficient in MLH1 or MSH6. *Proc Natl Acad Sci U S A* **98**, 13802–13807.

6. Davisson, M.T. (2003) Mitotic Chromosome Preparations from Bone Marrow. The Jackson Laboratory Website (http://www.jax.org/cyto/marrow_preps_alt.html).

7. Lichter, P., Ledbetter, S.A., Ledbetter, D.H., and Ward, D.C. (1990) Fluorescence in situ hybridization with Alu and L1 polymerase chain reaction probes for rapid characterization of human chromosomes in hybrid cell lines. *Proc Natl Acad Sci U S A* **87**, 6634–6638.

8. Yan, B., Wang, H., Wang, H., et al. (2006) Apoptotic DNA fragmentation factor maintains chromosome stability in a P53-independent manner. *Oncogene* **25**, 5370–5376.

9. Fenech, M. (2000) The in vitro micronucleus technique. *Mutat Res* **455**, 81–95.

10. de Souza-Pinto, N.C., Eide, L., Hogue, B.A., Thybo, T., Stevnsner, T., Seeberg, E., Klungland, A., and Bohr, V.A. (2001) Repair of 8-oxodeoxyguanosine lesions in mitochondrial DNA depends on the oxoguanine DNA glycosylase (OGG1) gene and 8-oxoguanine accumulates in the mitochondrial DNA of OGG1-defective mice. *Cancer Res* **61**, 5378–5381.

11. Cox, A.D., and Der, C.J.D. (1994) Biological assays for cellular transformation. *Methods Enzymol* **238**, 277–294.

Exhibit 75

Hallmarks of Cancer: The Next Generation

Douglas Hanahan[1,2,*] and Robert A. Weinberg[3,*]
[1]The Swiss Institute for Experimental Cancer Research (ISREC), School of Life Sciences, EPFL, Lausanne CH-1015, Switzerland
[2]The Department of Biochemistry & Biophysics, UCSF, San Francisco, CA 94158, USA
[3]Whitehead Institute for Biomedical Research, Ludwig/MIT Center for Molecular Oncology, and MIT Department of Biology, Cambridge, MA 02142, USA
*Correspondence: dh@epfl.ch (D.H.), weinberg@wi.mit.edu (R.A.W.)
DOI 10.1016/j.cell.2011.02.013

The hallmarks of cancer comprise six biological capabilities acquired during the multistep development of human tumors. The hallmarks constitute an organizing principle for rationalizing the complexities of neoplastic disease. They include sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis. Underlying these hallmarks are genome instability, which generates the genetic diversity that expedites their acquisition, and inflammation, which fosters multiple hallmark functions. Conceptual progress in the last decade has added two emerging hallmarks of potential generality to this list—reprogramming of energy metabolism and evading immune destruction. In addition to cancer cells, tumors exhibit another dimension of complexity: they contain a repertoire of recruited, ostensibly normal cells that contribute to the acquisition of hallmark traits by creating the "tumor microenvironment." Recognition of the widespread applicability of these concepts will increasingly affect the development of new means to treat human cancer.

INTRODUCTION

We have proposed that six hallmarks of cancer together constitute an organizing principle that provides a logical framework for understanding the remarkable diversity of neoplastic diseases (Hanahan and Weinberg, 2000). Implicit in our discussion was the notion that as normal cells evolve progressively to a neoplastic state, they acquire a succession of these hallmark capabilities, and that the multistep process of human tumor pathogenesis could be rationalized by the need of incipient cancer cells to acquire the traits that enable them to become tumorigenic and ultimately malignant.

We noted as an ancillary proposition that tumors are more than insular masses of proliferating cancer cells. Instead, they are complex tissues composed of multiple distinct cell types that participate in heterotypic interactions with one another. We depicted the recruited normal cells, which form tumor-associated stroma, as active participants in tumorigenesis rather than passive bystanders; as such, these stromal cells contribute to the development and expression of certain hallmark capabilities. During the ensuing decade this notion has been solidified and extended, revealing that the biology of tumors can no longer be understood simply by enumerating the traits of the cancer cells but instead must encompass the contributions of the "tumor microenvironment" to tumorigenesis.

In the course of remarkable progress in cancer research subsequent to this publication, new observations have served both to clarify and to modify the original formulation of the hallmark capabilities. In addition, yet other observations have raised questions and highlighted mechanistic concepts that were not integral to our original elaboration of the hallmark traits. Moti-

vated by these developments, we now revisit the original hallmarks, consider new ones that might be included in this roster, and expand upon the functional roles and contributions made by recruited stromal cells to tumor biology.

HALLMARK CAPABILITIES—CONCEPTUAL PROGRESS

The six hallmarks of cancer—distinctive and complementary capabilities that enable tumor growth and metastatic dissemination—continue to provide a solid foundation for understanding the biology of cancer (Figure 1; see the Supplemental Information for downloadable versions of the figures for presentations). In the first section of this Review, we summarize the essence of each hallmark as described in the original presentation in 2000, followed by selected illustrations (demarcated by subheadings in italics) of the conceptual progress made over the past decade in understanding their mechanistic underpinnings. In subsequent sections we address new developments that broaden the scope of the conceptualization, describing in turn two enabling characteristics crucial to the acquisition of the six hallmark capabilities, two new emerging hallmark capabilities, the constitution and signaling interactions of the tumor microenvironment crucial to cancer phenotypes, and we finally discuss the new frontier of therapeutic application of these concepts.

Sustaining Proliferative Signaling

Arguably the most fundamental trait of cancer cells involves their ability to sustain chronic proliferation. Normal tissues carefully control the production and release of growth-promoting signals that instruct entry into and progression through the cell growth-and-division cycle, thereby ensuring a homeostasis of cell

Cell
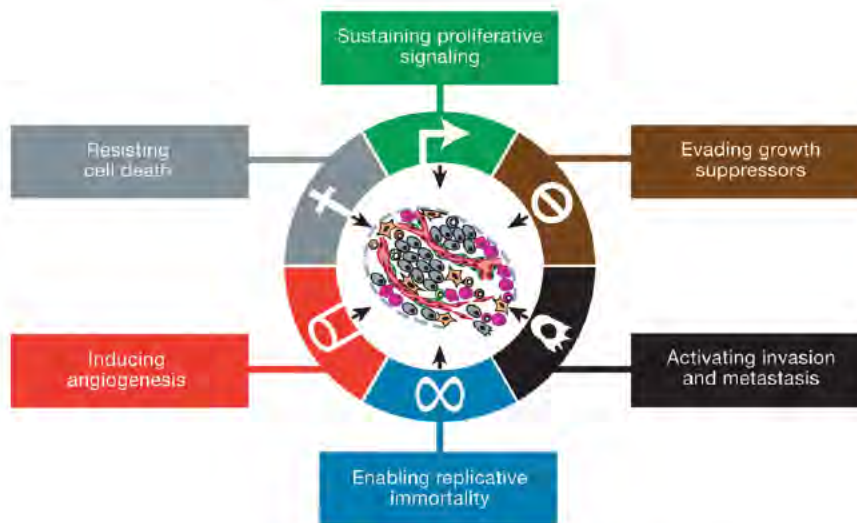
Figure 1. The Hallmarks of Cancer
This illustration encompasses the six hallmark capabilities originally proposed in our 2000 perspective. The past decade has witnessed remarkable progress toward understanding the mechanistic underpinnings of each hallmark.

number and thus maintenance of normal tissue architecture and function. Cancer cells, by deregulating these signals, become masters of their own destinies. The enabling signals are conveyed in large part by growth factors that bind cell-surface receptors, typically containing intracellular tyrosine kinase domains. The latter proceed to emit signals via branched intracellular signaling pathways that regulate progression through the cell cycle as well as cell growth (that is, increases in cell size); often these signals influence yet other cell-biological properties, such as cell survival and energy metabolism.

Remarkably, the precise identities and sources of the proliferative signals operating within normal tissues were poorly understood a decade ago and in general remain so. Moreover, we still know relatively little about the mechanisms controlling the release of these mitogenic signals. In part, the understanding of these mechanisms is complicated by the fact that the growth factor signals controlling cell number and position within tissues are thought to be transmitted in a temporally and spatially regulated fashion from one cell to its neighbors; such paracrine signaling is difficult to access experimentally. In addition, the bioavailability of growth factors is regulated by sequestration in the pericellular space and extracellular matrix, and by the actions of a complex network of proteases, sulfatases, and possibly other enzymes that liberate and activate them, apparently in a highly specific and localized fashion.

The mitogenic signaling in cancer cells is, in contrast, better understood (Lemmon and Schlessinger, 2010; Witsch et al., 2010; Hynes and MacDonald, 2009; Perona, 2006). Cancer cells can acquire the capability to sustain proliferative signaling in a number of alternative ways: They may produce growth factor ligands themselves, to which they can respond via the expression of cognate receptors, resulting in autocrine proliferative stimulation. Alternatively, cancer cells may send signals to stimulate normal cells within the supporting tumor-associated stroma, which reciprocate by supplying the cancer cells with various growth factors (Cheng et al., 2008; Bhowmick et al., 2004). Receptor signaling can also be deregulated by elevating the levels of receptor proteins displayed at the cancer cell

surface, rendering such cells hyperresponsive to otherwise-limiting amounts of growth factor ligand; the same outcome can result from structural alterations in the receptor molecules that facilitate ligand-independent firing.

Growth factor independence may also derive from the constitutive activation of components of signaling pathways operating downstream of these receptors, obviating the need to stimulate these pathways by ligand-mediated receptor activation. Given that a number of distinct downstream signaling pathways radiate from a ligand-stimulated receptor, the activation of one or another of these downstream pathways, for example, the one responding to the Ras signal transducer, may only recapitulate a subset of the regulatory instructions transmitted by an activated receptor.

Somatic Mutations Activate Additional Downstream Pathways

High-throughput DNA sequencing analyses of cancer cell genomes have revealed somatic mutations in certain human tumors that predict constitutive activation of signaling circuits usually triggered by activated growth factor receptors. Thus, we now know that ~40% of human melanomas contain activating mutations affecting the structure of the B-Raf protein, resulting in constitutive signaling through the Raf to mitogen-activated protein (MAP)-kinase pathway (Davies and Samuels 2010). Similarly, mutations in the catalytic subunit of phosphoinositide 3-kinase (PI3-kinase) isoforms are being detected in an array of tumor types, which serve to hyperactivate the PI3-kinase signaling circuitry, including its key Akt/PKB signal transducer (Jiang and Liu, 2009; Yuan and Cantley, 2008). The advantages to tumor cells of activating upstream (receptor) versus downstream (transducer) signaling remain obscure, as does the functional impact of crosstalk between the multiple pathways radiating from growth factor receptors.

Disruptions of Negative-Feedback Mechanisms that Attenuate Proliferative Signaling

Recent results have highlighted the importance of negative-feedback loops that normally operate to dampen various types of signaling and thereby ensure homeostatic regulation of the flux of signals coursing through the intracellular circuitry (Wertz and Dixit, 2010; Cabrita and Christofori, 2008; Amit et al., 2007; Mosesson et al., 2008). Defects in these feedback mechanisms are capable of enhancing proliferative signaling. The prototype of this type of regulation involves the Ras oncoprotein: the oncogenic effects of Ras do not result from a hyperactivation of its signaling powers; instead, the oncogenic mutations affecting ras genes compromise Ras GTPase activity, which

Cell

operates as an intrinsic negative-feedback mechanism that normally ensures that active signal transmission is transitory.

Analogous negative-feedback mechanisms operate at multiple nodes within the proliferative signaling circuitry. A prominent example involves the PTEN phosphatase, which counteracts PI3-kinase by degrading its product, phosphatidylinositol (3,4,5) trisphosphate (PIP_3). Loss-of-function mutations in PTEN amplify PI3K signaling and promote tumorigenesis in a variety of experimental models of cancer; in human tumors, PTEN expression is often lost by promoter methylation (Jiang and Liu, 2009; Yuan and Cantley, 2008).

Yet another example involves the mTOR kinase, a coordinator of cell growth and metabolism that lies both upstream and downstream of the PI3K pathway. In the circuitry of some cancer cells, mTOR activation results, via negative feedback, in the inhibition of PI3K signaling. Thus, when mTOR is pharmacologically inhibited in such cancer cells (such as by the drug rapamycin), the associated loss of negative feedback results in increased activity of PI3K and its effector Akt/PKB, thereby blunting the antiproliferative effects of mTOR inhibition (Sudarsanam and Johnson, 2010; O'Reilly et al., 2006). It is likely that compromised negative-feedback loops in this and other signaling pathways will prove to be widespread among human cancer cells and serve as an important means by which these cells can achieve proliferative independence. Moreover, disruption of such self-attenuating signaling may contribute to the development of adaptive resistance toward drugs targeting mitogenic signaling.

Excessive Proliferative Signaling Can Trigger Cell Senescence

Early studies of oncogene action encouraged the notion that ever-increasing expression of such genes and the signals manifested in their protein products would result in correspondingly increased cancer cell proliferation and thus tumor growth. More recent research has undermined this notion, in that excessively elevated signaling by oncoproteins such as RAS, MYC, and RAF can provoke counteracting responses from cells, specifically induction of cell senescence and/or apoptosis (Collado and Serrano, 2010; Evan and d'Adda di Fagagna, 2009; Lowe et al., 2004). For example, cultured cells expressing high levels of the Ras oncoprotein may enter into the nonproliferative but viable state called senescence; in contrast, cells expressing lower levels of this protein may avoid senescence and proliferate.

Cells with morphological features of senescence, including enlarged cytoplasm, the absence of proliferation markers, and expression of the senescence-induced β-galactosidase enzyme, are abundant in the tissues of mice engineered to overexpress certain oncogenes (Collado and Serrano, 2010; Evan and d'Adda di Fagagna, 2009) and are prevalent in some cases of human melanoma (Mooi and Peeper, 2006). These ostensibly paradoxical responses seem to reflect intrinsic cellular defense mechanisms designed to eliminate cells experiencing excessive levels of certain types of signaling. Accordingly, the relative intensity of oncogenic signaling in cancer cells may represent compromises between maximal mitogenic stimulation and avoidance of these antiproliferative defenses. Alternatively, some cancer cells may adapt to high levels of oncogenic signaling by disabling their senescence- or apoptosis-inducing circuitry.

Evading Growth Suppressors

In addition to the hallmark capability of inducing and sustaining positively acting growth-stimulatory signals, cancer cells must also circumvent powerful programs that negatively regulate cell proliferation; many of these programs depend on the actions of tumor suppressor genes. Dozens of tumor suppressors that operate in various ways to limit cell growth and proliferation have been discovered through their characteristic inactivation in one or another form of animal or human cancer; many of these genes have been validated as bona fide tumor suppressors through gain- or loss-of-function experiments in mice. The two prototypical tumor suppressors encode the RB (retinoblastoma-associated) and TP53 proteins; they operate as central control nodes within two key complementary cellular regulatory circuits that govern the decisions of cells to proliferate or, alternatively, activate senescence and apoptotic programs.

The RB protein integrates signals from diverse extracellular and intracellular sources and, in response, decides whether or not a cell should proceed through its growth-and-division cycle (Burkhart and Sage, 2008; Deshpande et al., 2005; Sherr and McCormick, 2002). Cancer cells with defects in RB pathway function are thus missing the services of a critical gatekeeper of cell-cycle progression whose absence permits persistent cell proliferation. Whereas RB transduces growth-inhibitory signals that originate largely outside of the cell, TP53 receives inputs from stress and abnormality sensors that function within the cell's intracellular operating systems: if the degree of damage to the genome is excessive, or if the levels of nucleotide pools, growth-promoting signals, glucose, or oxygenation are suboptimal, TP53 can call a halt to further cell-cycle progression until these conditions have been normalized. Alternatively, in the face of alarm signals indicating overwhelming or irreparable damage to such cellular subsystems, TP53 can trigger apoptosis. Notably, the various effects of activated TP53 are complex and highly context dependent, varying by cell type as well as by the severity and persistence of conditions of cell stress and genomic damage.

Although the two canonical suppressors of proliferation—TP53 and RB—have preeminent importance in regulating cell proliferation, various lines of evidence indicate that each operates as part of a larger network that is wired for functional redundancy. For example, chimeric mice populated throughout their bodies with individual cells lacking a functional *Rb* gene are surprisingly free of proliferative abnormalities, despite the expectation that loss of RB function would allow continuous firing of the cell division cycle in these cells and their lineal descendants; some of the resulting clusters of *Rb* null cells should, by all rights, progress to neoplasia. Instead, the *Rb* null cells in such chimeric mice have been found to participate in relatively normal tissue morphogenesis throughout the body; the only neoplasia observed was in the development of pituitary tumors late in life (Lipinski and Jacks, 1999). Similarly, *TP53* null mice develop normally, show largely proper cell and tissue homeostasis, and again develop abnormalities later in life, in the form of leukemias and sarcomas (Ghebranious and Donehower, 1998). Both examples must reflect the operations of redundantly acting mechanisms that serve to constrain inappropriate replication of cells lacking these key proliferation suppressors.

Cell

Mechanisms of Contact Inhibition and Its Evasion

Four decades of research have demonstrated that the cell-to-cell contacts formed by dense populations of normal cells propagated in two-dimensional culture operate to suppress further cell proliferation, yielding confluent cell monolayers. Importantly, such "contact inhibition" is abolished in various types of cancer cells in culture, suggesting that contact inhibition is an in vitro surrogate of a mechanism that operates in vivo to ensure normal tissue homeostasis, one that is abrogated during the course of tumorigenesis. Until recently, the mechanistic basis for this mode of growth control remained obscure. Now, however, mechanisms of contact inhibition are beginning to emerge.

One mechanism involves the product of the *NF2* gene, long implicated as a tumor suppressor because its loss triggers a form of human neurofibromatosis. Merlin, the cytoplasmic *NF2* gene product, orchestrates contact inhibition via coupling cell-surface adhesion molecules (e.g., E-cadherin) to transmembrane receptor tyrosine kinases (e.g., the EGF receptor). In so doing, Merlin strengthens the adhesivity of cadherin-mediated cell-to-cell attachments. Additionally, by sequestering growth factor receptors, Merlin limits their ability to efficiently emit mitogenic signals (Curto et al., 2007; Okada et al., 2005).

A second mechanism of contact inhibition involves the LKB1 epithelial polarity protein, which organizes epithelial structure and helps maintain tissue integrity. LKB1 can, for example, overrule the mitogenic effects of the powerful Myc oncogene when the latter is upregulated in organized, quiescent epithelial structures; in contrast, when LKB1 expression is suppressed, epithelial integrity is destabilized, and epithelial cells become susceptible to Myc-induced transformation (Partanen et al., 2009; Hezel and Bardeesy, 2008). *LKB1* has also been identified as a tumor suppressor gene that is lost in certain human malignancies (Shaw, 2009), possibly reflecting its normal function as a suppressor of inappropriate proliferation. It remains to be seen how frequently these two mechanisms of contact-mediated growth suppression are compromised in human cancers; no doubt yet other contact-induced proliferative barriers are yet to be discovered. Clearly mechanisms like these that enable cells to construct and maintain architecturally complex tissues represent important means of suppressing and counterbalancing inappropriate proliferative signals.

Corruption of the TGF-β Pathway Promotes Malignancy

TGF-β is best known for its antiproliferative effects, and evasion by cancer cells of these effects is now appreciated to be far more elaborate than simple shutdown of its signaling circuitry (Ikushima and Miyazono, 2010; Massagué, 2008; Bierie and Moses, 2006). In many late-stage tumors, TGF-β signaling is redirected away from suppressing cell proliferation and is found instead to activate a cellular program, termed the epithelial-to-mesenchymal transition (EMT), that confers on cancer cells traits associated with high-grade malignancy, as discussed in further detail below.

Resisting Cell Death

The concept that programmed cell death by apoptosis serves as a natural barrier to cancer development has been established by compelling functional studies conducted over the last two decades (Adams and Cory, 2007; Lowe et al., 2004: Evan and

Littlewood, 1998). Elucidation of the signaling circuitry governing the apoptotic program has revealed how apoptosis is triggered in response to various physiologic stresses that cancer cells experience during the course of tumorigenesis or as a result of anticancer therapy. Notable among the apoptosis-inducing stresses are signaling imbalances resulting from elevated levels of oncogene signaling, as mentioned earlier, and DNA damage associated with hyperproliferation. Yet other research has revealed how apoptosis is attenuated in those tumors that succeed in progressing to states of high-grade malignancy and resistance to therapy (Adams and Cory, 2007; Lowe et al., 2004).

The apoptotic machinery is composed of both upstream regulators and downstream effector components (Adams and Cory, 2007). The regulators, in turn, are divided into two major circuits, one receiving and processing extracellular death-inducing signals (the extrinsic apoptotic program, involving for example the Fas ligand/Fas receptor), and the other sensing and integrating a variety of signals of intracellular origin (the intrinsic program). Each culminates in activation of a normally latent protease (caspases 8 and 9, respectively), which proceeds to initiate a cascade of proteolysis involving effector caspases responsible for the execution phase of apoptosis, in which the cell is progressively disassembled and then consumed, both by its neighbors and by professional phagocytic cells. Currently, the intrinsic apoptotic program is more widely implicated as a barrier to cancer pathogenesis.

The "apoptotic trigger" that conveys signals between the regulators and effectors is controlled by counterbalancing pro- and antiapoptotic members of the Bcl-2 family of regulatory proteins (Adams and Cory, 2007). The archetype, Bcl-2, along with its closest relatives (Bcl-x$_L$, Bcl-w, Mcl-1, A1) are inhibitors of apoptosis, acting in large part by binding to and thereby suppressing two proapoptotic triggering proteins (Bax and Bak); the latter are embedded in the mitochondrial outer membrane. When relieved of inhibition by their antiapoptotic relatives, Bax and Bak disrupt the integrity of the outer mitochondrial membrane, causing the release of proapoptotic signaling proteins, the most important of which is cytochrome *c*. The released cytochrome *c* activates, in turn, a cascade of caspases that act via their proteolytic activities to induce the multiple cellular changes associated with the apoptotic program. Bax and Bak share protein-protein interaction domains, termed BH3 motifs, with the antiapoptotic Bcl-2-like proteins that mediate their various physical interactions. The activities of a subfamily of related proteins, each of which contains a single such BH3 motif, are coupled to a variety of sensors of cellular abnormality; these "BH3-only" proteins act either by interfering with antiapoptotic Bcl-2 proteins or by directly stimulating the proapoptotic members of this family (Adams and Cory, 2007; Willis and Adams, 2005).

Although the cellular conditions that trigger apoptosis remain to be fully enumerated, several abnormality sensors that play key roles in tumor development have been identified (Adams and Cory, 2007; Lowe et al., 2004). Most notable is a DNA-damage sensor that functions via the TP53 tumor suppressor (Junttila and Evan, 2009); TP53 induces apoptosis by upregulating expression of the Noxa and Puma BH3-only proteins, doing so in response to substantial levels of DNA breaks and other chromosomal abnormalities. Alternatively, insufficient survival

Cell

factor signaling (for instance inadequate levels of interleukin-3 in lymphocytes or of insulin-like growth factor 1/2 [Igf1/2] in epithelial cells) can elicit apoptosis through a BH3-only protein called Bim. Yet another condition leading to cell death involves hyperactive signaling by certain oncoproteins, such as Myc, which triggers apoptosis (in part via Bim and other BH3-only proteins) unless counterbalanced by antiapoptotic factors (Junttila and Evan, 2009; Lowe et al., 2004).

Tumor cells evolve a variety of strategies to limit or circumvent apoptosis. Most common is the loss of TP53 tumor suppressor function, which eliminates this critical damage sensor from the apoptosis-inducing circuitry. Alternatively, tumors may achieve similar ends by increasing expression of antiapoptotic regulators (Bcl-2, Bcl-x$_L$) or of survival signals (Igf1/2), by downregulating proapoptotic factors (Bax, Bim, Puma), or by short-circuiting the extrinsic ligand-induced death pathway. The multiplicity of apoptosis-avoiding mechanisms presumably reflects the diversity of apoptosis-inducing signals that cancer cell populations encounter during their evolution to the malignant state.

The structure of the apoptotic machinery and program, and the strategies used by cancer cells to evade its actions, were widely appreciated by the beginning of the last decade. The most notable conceptual advances since then have involved other forms of cell death that broaden the scope of "programmed cell death" as a barrier to cancer.

Autophagy Mediates Both Tumor Cell Survival and Death

Autophagy represents an important cell-physiologic response that, like apoptosis, normally operates at low, basal levels in cells but can be strongly induced in certain states of cellular stress, the most obvious of which is nutrient deficiency (Levine and Kroemer, 2008; Mizushima, 2007). The autophagic program enables cells to break down cellular organelles, such as ribosomes and mitochondria, allowing the resulting catabolites to be recycled and thus used for biosynthesis and energy metabolism. As part of this program, intracellular vesicles termed autophagosomes envelope intracellular organelles and then fuse with lysosomes wherein degradation occurs. In this fashion, low-molecular-weight metabolites are generated that support survival in the stressed, nutrient-limited environments experienced by many cancer cells.

Like apoptosis, the autophagy machinery has both regulatory and effector components (Levine and Kroemer, 2008; Mizushima, 2007). Among the latter are proteins that mediate autophagosome formation and delivery to lysosomes. Of note, recent research has revealed intersections between the regulatory circuits governing autophagy, apoptosis, and cellular homeostasis. For example, the signaling pathway involving the PI3-kinase, AKT, and mTOR kinases, which is stimulated by survival signals to block apoptosis, similarly inhibits autophagy; when survival signals are insufficient, the PI3K signaling pathway is downregulated, with the result that autophagy and/or apoptosis may be induced (Levine and Kroemer, 2008; Sinha and Levine, 2008; Mathew et al., 2007).

Another interconnection between these two programs resides in the Beclin-1 protein, which has been shown by genetic studies to be necessary for induction of autophagy (Levine and Kroemer, 2008; Sinha and Levine, 2008; Mizushima, 2007). Beclin-1 is a member of the BH3-only subfamily of apoptotic regulatory

proteins, and its BH3 domain allows it to bind the Bcl-2/Bcl-x$_L$ proteins. Stress-sensor-coupled BH3 proteins can displace Beclin-1 from its association with Bcl-2/Bcl-x$_L$, enabling the liberated Beclin-1 to trigger autophagy, much as they can release proapoptotic Bax and Bak to trigger apoptosis. Hence, stress-transducing BH3 proteins (e.g., Bid, Bad, Puma, et al.) can induce apoptosis and/or autophagy depending on the physiologic state of the cell.

Mice bearing inactivated alleles of the *Beclin-1* gene or of certain other components of the autophagy machinery exhibit increased susceptibility to cancer (White and DiPaola, 2009; Levine and Kroemer, 2008). These results suggest that induction of autophagy can serve as a barrier to tumorigenesis that may operate independently of or in concert with apoptosis. Accordingly, autophagy appears to represent yet another barrier that needs to be circumvented during tumor development (White and DiPaola, 2009).

Perhaps paradoxically, nutrient starvation, radiotherapy, and certain cytotoxic drugs can induce elevated levels of autophagy that are apparently cytoprotective for cancer cells, impairing rather than accentuating the killing actions of these stress-inducing situations (White and DiPaola, 2009; Apel et al., 2009; Amaravadi and Thompson, 2007; Mathew et al., 2007). Moreover, severely stressed cancer cells have been shown to shrink via autophagy to a state of reversible dormancy (White and DiPaola, 2009; Lu et al., 2008). This survival response may enable the persistence and eventual regrowth of some late-stage tumors following treatment with potent anticancer agents. Thus, in analogy to TGF-β signaling, which can be tumor suppressing at early stages of tumorigenesis and tumor promoting later on, autophagy seems to have conflicting effects on tumor cells and thus tumor progression (Apel et al., 2009; White and DiPaola, 2009). An important agenda for future research will involve clarifying the genetic and cell-physiologic conditions that dictate when and how autophagy enables cancer cells to survive or causes them to die.

Necrosis Has Proinflammatory and Tumor-Promoting Potential

In contrast to apoptosis, in which a dying cell contracts into an almost-invisible corpse that is soon consumed by neighbors, necrotic cells become bloated and explode, releasing their contents into the local tissue microenvironment. Although necrosis has historically been viewed much like organismic death, as a form of system-wide exhaustion and breakdown, the conceptual landscape is changing: cell death by necrosis is clearly under genetic control in some circumstances, rather than being a random and undirected process (Galluzzi and Kroemer, 2008; Zong and Thompson, 2006).

Perhaps more important, necrotic cell death releases proinflammatory signals into the surrounding tissue microenvironment, in contrast to apoptosis and autophagy, which do not. As a consequence, necrotic cells can recruit inflammatory cells of the immune system (Grivennikov et al., 2010; White et al., 2010; Galluzzi and Kroemer, 2008), whose dedicated function is to survey the extent of tissue damage and remove associated necrotic debris. In the context of neoplasia, however, multiple lines of evidence indicate that immune inflammatory cells can be actively tumor promoting, given that such cells are capable

Cell

of fostering angiogenesis, cancer cell proliferation, and invasiveness (see below). Additionally, necrotic cells can release bioactive regulatory factors, such as IL-1α, which can directly stimulate neighboring viable cells to proliferate, with the potential, once again, to facilitate neoplastic progression (Grivennikov et al., 2010). Consequently, necrotic cell death, while seemingly beneficial in counterbalancing cancer-associated hyperproliferation, may ultimately do more damage than good. Accordingly, incipient neoplasias and potentially invasive and metastatic tumors may gain an advantage by tolerating some degree of necrotic cell death, doing so in order to recruit tumor-promoting inflammatory cells that bring growth-stimulating factors to the surviving cells within these growths.

Enabling Replicative Immortality

By 2000, it was widely accepted that cancer cells require unlimited replicative potential in order to generate macroscopic tumors. This capability stands in marked contrast to the behavior of the cells in most normal cell lineages in the body, which are able to pass through only a limited number of successive cell growth-and-division cycles. This limitation has been associated with two distinct barriers to proliferation: senescence, a typically irreversible entrance into a nonproliferative but viable state, and crisis, which involves cell death. Accordingly, when cells are propagated in culture, repeated cycles of cell division lead first to induction of senescence and then, for those cells that succeed in circumventing this barrier, to a crisis phase, in which the great majority of cells in the population die. On rare occasion, cells emerge from a population in crisis and exhibit unlimited replicative potential. This transition has been termed immortalization, a trait that most established cell lines possess by virtue of their ability to proliferate in culture without evidence of either senescence or crisis.

Multiple lines of evidence indicate that telomeres protecting the ends of chromosomes are centrally involved in the capability for unlimited proliferation (Blasco, 2005; Shay and Wright, 2000). The telomeres, composed of multiple tandem hexanucleotide repeats, shorten progressively in nonimmortalized cells propagated in culture, eventually losing the ability to protect the ends of chromosomal DNAs from end-to-end fusions; such fusions generate unstable dicentric chromosomes whose resolution results in a scrambling of karyotype that threatens cell viability. Accordingly, the length of telomeric DNA in a cell dictates how many successive cell generations its progeny can pass through before telomeres are largely eroded and have consequently lost their protective functions, triggering entrance into crisis.

Telomerase, the specialized DNA polymerase that adds telomere repeat segments to the ends of telomeric DNA, is almost absent in nonimmortalized cells but expressed at functionally significant levels in the vast majority (~90%) of spontaneously immortalized cells, including human cancer cells. By extending telomeric DNA, telomerase is able to counter the progressive telomere erosion that would otherwise occur in its absence. The presence of telomerase activity, either in spontaneously immortalized cells or in the context of cells engineered to express the enzyme, is correlated with a resistance to induction of both senescence and crisis/apoptosis; conversely, suppression of telomerase activity leads to telomere shortening and to activation of one or the other of these proliferative barriers.

The two barriers to proliferation—senescence and crisis/apoptosis—have been rationalized as crucial anticancer defenses that are hard-wired into our cells, being deployed to impede the outgrowth of clones of preneoplastic and frankly neoplastic cells. According to this thinking, most incipient neoplasias exhaust their endowment of replicative doublings and are stopped in their tracks by one or the other of these barriers. The eventual immortalization of rare variant cells that proceed to form tumors has been attributed to their ability to maintain telomeric DNA at lengths sufficient to avoid triggering senescence or apoptosis, achieved most commonly by upregulating expression of telomerase or, less frequently, via an alternative recombination-based telomere maintenance mechanism. Hence, telomere shortening has come to be viewed as a clocking device that determines the limited replicative potential of normal cells and thus one that must be overcome by cancer cells.

Reassessing Replicative Senescence

Whereas telomere maintenance has been increasingly substantiated as a condition critical to the neoplastic state, the concept of replication-induced senescence as a general barrier requires refinement and reformulation. (Differences in telomere structure and function in mouse versus human cells have also complicated investigation of the roles of telomeres and telomerase in replicative senescence.) Recent experiments have revealed that the induction of senescence in certain cultured cells can be delayed and possibly eliminated by the use of improved cell culture conditions, suggesting that recently explanted primary cells may be able to proliferate unimpeded in culture up the point of crisis and the associated induction of apoptosis triggered by critically shortened telomeres (Ince et al., 2007; Passos et al., 2007; Zhang et al., 2004; Sherr and DePinho, 2000). In contrast, experiments in mice engineered to lack telomerase indicate that the consequently shortened telomeres can shunt premalignant cells into a senescent state that contributes (along with apoptosis) to attenuated tumorigenesis in mice genetically destined to develop particular forms of cancer (Artandi and DePinho, 2010). Such telomerase null mice with highly eroded telomeres exhibit multiorgan dysfunction and abnormalities that include evidence for both senescence and apoptosis, perhaps analogous to the senescence and apoptosis observed in cell culture (Artandi and DePinho, 2010; Feldser and Greider, 2007).

Of note, and as discussed earlier, a morphologically similar form of cell senescence induced by excessive or unbalanced oncogene signaling is now well documented as a protective mechanism against neoplasia; the possible interconnections of this form of senescence with telomerase and telomeres remain to be ascertained. Thus, cell senescence is emerging conceptually as a protective barrier to neoplastic expansion that can be triggered by various proliferation-associated abnormalities, including high levels of oncogenic signaling and, apparently, subcritical shortening of telomeres.

Delayed Activation of Telomerase May Both Limit and Foster Neoplastic Progression

There is now evidence that clones of incipient cancer cells often experience telomere loss-induced crisis relatively early during

Cell

the course of multistep tumor progression due to their inability to express significant levels of telomerase. Thus, extensively eroded telomeres have been documented in premalignant growths through the use of fluorescence in situ hybridization (FISH), which has also revealed the end-to-end chromosomal fusions that signal telomere failure and crisis (Kawai et al., 2007; Hansel et al., 2006). These results also suggest that such cells have passed through a substantial number of successive telomere-shortening cell divisions during their evolution from fully normal cells-of-origin. Accordingly, the development of some human neoplasias may be aborted by telomere-induced crisis long before they succeed in becoming macroscopic, frankly neoplastic growths.

In contrast, the absence of TP53-mediated surveillance of genomic integrity may permit other incipient neoplasias to survive initial telomere erosion and attendant chromosomal breakage-fusion-bridge (BFB) cycles. The genomic alterations resulting from these BFB cycles, including deletions and amplifications of chromosomal segments, evidently serve to increase the mutability of the genome, thereby accelerating the acquisition of mutant oncogenes and tumor suppressor genes. The realization that impaired telomere function can actually foster tumor progression has come from the study of mutant mice that lack both p53 and telomerase function (Artandi and DePinho, 2010, 2000). The proposition that these two defects can cooperatively enhance human tumorigenesis has not yet been directly documented.

Circumstantial support for the importance of transient telomere deficiency in facilitating malignant progression has come, in addition, from comparative analyses of premalignant and malignant lesions in the human breast (Raynaud et al., 2010; Chin et al., 2004). The premalignant lesions did not express significant levels of telomerase and were marked by telomere shortening and nonclonal chromosomal aberrations. In contrast, overt carcinomas exhibited telomerase expression concordantly with the reconstruction of longer telomeres and the fixation (via clonal outgrowth) of the aberrant karyotypes that would seem to have been acquired after telomere failure but before the acquisition of telomerase activity. When portrayed in this way, the delayed acquisition of telomerase function serves to generate tumor-promoting mutations, whereas its subsequent activation stabilizes the mutant genome and confers the unlimited replicative capacity that cancer cells require in order to generate clinically apparent tumors.

New Functions of Telomerase

Telomerase was discovered because of its ability to elongate and maintain telomeric DNA, and almost all telomerase research has been posited on the notion that its functions are confined to this crucial function. However, in recent years it has become apparent that telomerase exerts functions that are relevant to cell proliferation but unrelated to telomere maintenance. The noncanonical roles of telomerase, and in particular its protein subunit TERT, have been revealed by functional studies in mice and cultured cells; in some cases novel functions have been demonstrated in conditions where the telomerase enzymatic activity has been eliminated (Cong and Shay, 2008). Among the growing list of telomere-independent functions of TERT/telomerase is the ability of TERT to amplify signaling by

the Wnt pathway, by serving as a cofactor of the β-catenin/LEF transcription factor complex (Park et al., 2009). Other ascribed telomere-independent effects include demonstrable enhancement of cell proliferation and/or resistance to apoptosis (Kang et al., 2004), involvement in DNA-damage repair (Masutomi et al., 2005), and RNA-dependent RNA polymerase function (Maida et al., 2009). Consistent with these broader roles, TERT can be found associated with chromatin at multiple sites along the chromosomes, not just at the telomeres (Park et al., 2009; Masutomi et al., 2005). Hence, telomere maintenance is proving to be the most prominent of a diverse series of functions to which TERT contributes. The contributions of these additional functions of telomerase to tumorigenesis remain to be fully elucidated.

Inducing Angiogenesis

Like normal tissues, tumors require sustenance in the form of nutrients and oxygen as well as an ability to evacuate metabolic wastes and carbon dioxide. The tumor-associated neovasculature, generated by the process of angiogenesis, addresses these needs. During embryogenesis, the development of the vasculature involves the birth of new endothelial cells and their assembly into tubes (vasculogenesis) in addition to the sprouting (angiogenesis) of new vessels from existing ones. Following this morphogenesis, the normal vasculature becomes largely quiescent. In the adult, as part of physiologic processes such as wound healing and female reproductive cycling, angiogenesis is turned on, but only transiently. In contrast, during tumor progression, an "angiogenic switch" is almost always activated and remains on, causing normally quiescent vasculature to continually sprout new vessels that help sustain expanding neoplastic growths (Hanahan and Folkman, 1996).

A compelling body of evidence indicates that the angiogenic switch is governed by countervailing factors that either induce or oppose angiogenesis (Baeriswyl and Christofori, 2009; Bergers and Benjamin, 2003). Some of these angiogenic regulators are signaling proteins that bind to stimulatory or inhibitory cell-surface receptors displayed by vascular endothelial cells. The well-known prototypes of angiogenesis inducers and inhibitors are vascular endothelial growth factor-A (VEGF-A) and thrombospondin-1 (TSP-1), respectively.

The VEGF-A gene encodes ligands that are involved in orchestrating new blood vessel growth during embryonic and postnatal development, and then in homeostatic survival of endothelial cells, as well as in physiological and pathological situations in the adult. VEGF signaling via three receptor tyrosine kinases (VEGFR-1–3) is regulated at multiple levels, reflecting this complexity of purpose. Thus, VEGF gene expression can by upregulated both by hypoxia and by oncogene signaling (Ferrara, 2009; Mac Gabhann and Popel, 2008; Carmeliet, 2005). Additionally, VEGF ligands can be sequestered in the extracellular matrix in latent forms that are subject to release and activation by extracellular matrix-degrading proteases (e.g., MMP-9; Kessenbrock et al., 2010). In addition, other proangiogenic signals, such as members of the fibroblast growth factor (FGF) family, have been implicated in sustaining tumor angiogenesis when their expression is chronically upregulated (Baeriswyl and Christofori, 2009). TSP-1, a key counterbalance in the

Cell

angiogenic switch, also binds transmembrane receptors displayed by endothelial cells and thereby evokes suppressive signals that can counteract proangiogenic stimuli (Kazerounian et al., 2008).

The blood vessels produced within tumors by chronically activated angiogenesis and an unbalanced mix of proangiogenic signals are typically aberrant: tumor neovasculature is marked by precocious capillary sprouting, convoluted and excessive vessel branching, distorted and enlarged vessels, erratic blood flow, microhemorrhaging, leakiness, and abnormal levels of endothelial cell proliferation and apoptosis (Nagy et al., 2010; Baluk et al., 2005).

Angiogenesis is induced surprisingly early during the multistage development of invasive cancers both in animal models and in humans. Histological analyses of premalignant, noninvasive lesions, including dysplasias and in situ carcinomas arising in a variety of organs, have revealed the early tripping of the angiogenic switch (Raica et al., 2009; Hanahan and Folkman, 1996). Historically, angiogenesis was envisioned to be important only when rapidly growing macroscopic tumors had formed, but more recent data indicate that angiogenesis also contributes to the microscopic premalignant phase of neoplastic progression, further cementing its status as an integral hallmark of cancer.

The past decade has witnessed an astonishing outpouring of research on angiogenesis. Amid this wealth of new knowledge, we highlight several advances of particular relevance to tumor physiology.

Gradations of the Angiogenic Switch
Once angiogenesis has been activated, tumors exhibit diverse patterns of neovascularization. Some tumors, including such highly aggressive types as pancreatic ductal adenocarcinomas, are hypovascularized and replete with stromal "deserts" that are largely avascular and indeed may even be actively antiangiogenic (Olive et al., 2009). Many other tumors, including human renal and pancreatic neuroendocrine carcinomas, are highly angiogenic and consequently densely vascularized (Zee et al., 2010; Turner et al., 2003).

Collectively, such observations suggest an initial tripping of the angiogenic switch during tumor development that is followed by a variable intensity of ongoing neovascularization, the latter being controlled by a complex biological rheostat that involves both the cancer cells and the associated stromal microenvironment (Baeriswyl and Christofori, 2009; Bergers and Benjamin, 2003). Of note, the switching mechanism can vary in its form, even though the net result is a common inductive signal (e.g., VEGF). In some tumors, dominant oncogenes operating within tumor cells, such as *Ras* and *Myc*, can upregulate expression of angiogenic factors, whereas in others, such inductive signals are produced indirectly by immune inflammatory cells, as discussed below. The direct induction of angiogenesis by oncogenes that also drive proliferative signaling illustrates the important principle that distinct hallmark capabilities can be coregulated by the same transforming agents.

Endogenous Angiogenesis Inhibitors Present Natural Barriers to Tumor Angiogenesis
Research in the 1990s revealed that TSP-1 as well as fragments of plasmin (angiostatin) and type 18 collagen (endostatin) can act as endogenous inhibitors of angiogenesis (Ribatti, 2009; Kazerounian, et al., 2008; Folkman, 2006, 2002; Nyberg et al., 2005). The last decade has seen reports of another dozen such agents (Ribatti, 2009; Folkman, 2006; Nyberg et al., 2005). Most are proteins, and many are derived by proteolytic cleavage of structural proteins that are not themselves angiogenic regulators. A number of these endogenous inhibitors of angiogenesis can be detected in the circulation of normal mice and humans. The genes encoding several endogenous angiogenesis inhibitors have been deleted from the mouse germline without untoward physiological effects; the growth of autochthonous and implanted tumors, however, is enhanced as a consequence (Ribatti, 2009; Nyberg et al., 2005). By contrast, if the circulating levels of an endogenous inhibitor are genetically increased (e.g., via overexpression in transgenic mice or in xenotransplanted tumors), tumor growth is impaired (Ribatti, 2009; Nyberg et al., 2005); interestingly, wound healing and fat deposition are impaired or accelerated by elevated or ablated expression of such genes (Cao, 2010; Seppinen et al., 2008). The data suggest that such endogenous angiogenesis inhibitors serve under normal circumstances as physiologic regulators that modulate transitory angiogenesis during tissue remodeling and wound healing; they may also act as intrinsic barriers to induction and/or persistence of angiogenesis by incipient neoplasias.

Pericytes Are Important Components of the Tumor Neovasculature
Pericytes have long been known as supporting cells that are closely apposed to the outer surfaces of the endothelial tubes in normal tissue vasculature, where they provide important mechanical and physiologic support to the endothelial cells. Tumor-associated vasculature, in contrast, was portrayed as lacking appreciable coverage by these auxiliary cells. However, careful microscopic studies conducted in recent years have revealed that pericytes are associated, albeit loosely, with the neovasculature of most if not all tumors (Raza et al., 2010; Bergers and Song, 2005). More importantly, mechanistic studies discussed below have revealed that pericyte coverage is important for the maintenance of a functional tumor neovasculature.

A Variety of Bone Marrow-Derived Cells Contribute to Tumor Angiogenesis
It is now clear that a repertoire of cell types originating in the bone marrow play crucial roles in pathological angiogenesis (Qian and Pollard, 2010; Zumsteg and Christofori, 2009; Murdoch et al., 2008; De Palma et al., 2007). These include cells of the innate immune system—notably macrophages, neutrophils, mast cells, and myeloid progenitors—that infiltrate premalignant lesions and progressed tumors and assemble at the margins of such lesions; the peri-tumoral inflammatory cells help to trip the angiogenic switch in previously quiescent tissue and to sustain ongoing angiogenesis associated with tumor growth, in addition to facilitating local invasion, as noted below. In addition, they can help protect the vasculature from the effects of drugs targeting endothelial cell signaling (Ferrara, 2010). Additionally, several types of bone marrow-derived "vascular progenitor cells" have been observed in certain cases to have migrated into neoplastic lesions and become intercalated into the neovasculature as pericytes or endothelial cells (Patenaude et al., 2010; Kovacic and Boehm, 2009; Lamagna and Bergers, 2006).

Cell

Activating Invasion and Metastasis

In 2000, the mechanisms underlying invasion and metastasis were largely an enigma. It was clear that as carcinomas arising from epithelial tissues progressed to higher pathological grades of malignancy, reflected in local invasion and distant metastasis, the associated cancer cells typically developed alterations in their shape as well as in their attachment to other cells and to the extracellular matrix (ECM). The best characterized alteration involved the loss by carcinoma cells of E-cadherin, a key cell-to-cell adhesion molecule. By forming adherens junctions with adjacent epithelial cells, E-cadherin helps to assemble epithelial cell sheets and maintain the quiescence of the cells within these sheets. Increased expression of E-cadherin was well established as an antagonist of invasion and metastasis, whereas reduction of its expression was known to potentiate these phenotypes. The frequently observed downregulation and occasional mutational inactivation of E-cadherin in human carcinomas provided strong support for its role as a key suppressor of this hallmark capability (Berx and van Roy, 2009; Cavallaro and Christofori, 2004).

Additionally, expression of genes encoding other cell-to-cell and cell-to-ECM adhesion molecules is demonstrably altered in some highly aggressive carcinomas, with those favoring cytostasis typically being downregulated. Conversely, adhesion molecules normally associated with the cell migrations that occur during embryogenesis and inflammation are often upregulated. For example, N-cadherin, which is normally expressed in migrating neurons and mesenchymal cells during organogenesis, is upregulated in many invasive carcinoma cells. Beyond the gain and loss of such cell-cell/matrix attachment proteins, the master regulators of invasion and metastasis were largely unknown or, when suspected, lacking in functional validation (Cavallaro and Christofori, 2004).

The multistep process of invasion and metastasis has been schematized as a sequence of discrete steps, often termed the invasion-metastasis cascade (Talmadge and Fidler, 2010; Fidler, 2003). This depiction envisions a succession of cell-biologic changes, beginning with local invasion, then intravasation by cancer cells into nearby blood and lymphatic vessels, transit of cancer cells through the lymphatic and hematogenous systems, followed by escape of cancer cells from the lumina of such vessels into the parenchyma of distant tissues (extravasation), the formation of small nodules of cancer cells (micrometastases), and finally the growth of micrometastatic lesions into macroscopic tumors, this last step being termed "colonization."

Research into the capability for invasion and metastasis has accelerated dramatically over the past decade as powerful new research tools and refined experimental models have become available, and as critical regulatory genes were identified. While still an emerging field replete with major unanswered questions, significant progress has been made in delineating important features of this complex hallmark capability. An admittedly incomplete representation of these advances is highlighted below.

The EMT Program Broadly Regulates Invasion and Metastasis

A developmental regulatory program, referred to as the "epithelial-mesenchymal transition" (EMT), has become prominently implicated as a means by which transformed epithelial cells can acquire the abilities to invade, to resist apoptosis, and to disseminate (Klymkowsky and Savagner, 2009; Polyak and Weinberg, 2009; Thiery et al., 2009; Yilmaz and Christofori, 2009; Barrallo-Gimeno and Nieto, 2005). By co-opting a process involved in various steps of embryonic morphogenesis and wound healing, carcinoma cells can concomitantly acquire multiple attributes that enable invasion and metastasis. This multifaceted EMT program can be activated transiently or stably, and to differing degrees, by carcinoma cells during the course of invasion and metastasis.

A set of pleiotropically acting transcriptional factors, including Snail, Slug, Twist, and Zeb1/2, orchestrate the EMT and related migratory processes during embryogenesis; most were initially identified by developmental genetics. These transcriptional regulators are expressed in various combinations in a number of malignant tumor types and have been shown in experimental models of carcinoma formation to be causally important for programming invasion; some have been found to elicit metastasis when ectopically overexpressed (Micalizzi et al., 2010; Taube et al., 2010; Schmalhofer et al., 2009; Yang and Weinberg, 2008). Included among the cell-biological traits evoked by such transcription factors are loss of adherens junctions and associated conversion from a polygonal/epithelial to a spindly/fibroblastic morphology, expression of matrix-degrading enzymes, increased motility, and heightened resistance to apoptosis—all traits implicated in the processes of invasion and metastasis. Several of these transcription factors can directly repress E-cadherin gene expression, thereby depriving neoplastic epithelial cells of this key suppressor of motility and invasiveness (Peinado et al., 2004).

The available evidence suggests that these transcription factors regulate one another as well as overlapping sets of target genes. No rules have yet been established to describe their interactions and the conditions that govern their expression. Evidence from developmental genetics indicates that contextual signals received from neighboring cells in the embryo are involved in triggering expression of these transcription factors in those cells destined to pass through an EMT (Micalizzi et al., 2010); in an analogous fashion, increasing evidence suggests that heterotypic interactions of cancer cells with adjacent tumor-associated stromal cells can induce expression of the malignant cell phenotypes that are known to be choreographed by one or more of these transcriptional regulators (Karnoub and Weinberg, 2006–2007; Brabletz et al., 2001). Moreover, cancer cells at the invasive margins of certain carcinomas can be seen to have undergone an EMT, suggesting that these cancer cells are subject to microenvironmental stimuli distinct from those received by cancer cells located in the cores of these lesions (Hlubek et al., 2007).

Although the evidence is still incomplete, it would appear that EMT-inducing transcription factors are able to orchestrate most steps of the invasion-metastasis cascade save the final step of colonization. We still know rather little about the various manifestations and temporal stability of the mesenchymal state produced by an EMT. Although expression of EMT-inducing transcription factors has been observed in certain nonepithelial tumor types, such as sarcomas and neuroectodermal tumors, their roles in programming malignant traits in these tumors are

Cell

presently poorly documented. Additionally, it remains to be determined whether invasive carcinoma cells necessarily acquire their capability through activation of parts of the EMT program, or whether alternative regulatory programs can also enable this capability.

Heterotypic Contributions of Stromal Cells to Invasion and Metastasis

It is increasingly apparent that crosstalk between cancer cells and cells of the neoplastic stroma is involved in the acquired capability for invasive growth and metastasis (Egeblad et al., 2010; Qian and Pollard, 2010; Joyce and Pollard, 2009; Kalluri and Zeisberg, 2006). Such signaling may impinge on carcinoma cells and act to alter their hallmark capabilities as suggested above. For example, mesenchymal stem cells (MSCs) present in the tumor stroma have been found to secrete CCL5/RANTES in response to signals released by cancer cells; CCL5 then acts reciprocally on the cancer cells to stimulate invasive behavior (Karnoub et al., 2007).

Macrophages at the tumor periphery can foster local invasion by supplying matrix-degrading enzymes such as metalloproteinases and cysteine cathepsin proteases (Kessenbrock et al., 2010; Joyce and Pollard, 2009; Palermo and Joyce, 2008; Mohamed and Sloane, 2006); in one model system, the invasion-promoting macrophages are activated by IL-4 produced by the cancer cells (Gocheva et al., 2010). And in an experimental model of metastatic breast cancer, tumor-associated macrophages (TAMs) supply epidermal growth factor (EGF) to breast cancer cells, while the cancer cells reciprocally stimulate the macrophages with CSF-1; their concerted interactions facilitate intravasation into the circulatory system and metastatic dissemination of the cancer cells (Qian and Pollard, 2010; Wyckoff et al., 2007).

Observations like these indicate that the phenotypes of high-grade malignancy do not arise in a strictly cell-autonomous manner, and that their manifestation cannot be understood solely through analyses of tumor cell genomes. One important implication, still untested, is that the ability to negotiate most of the steps of the invasion-metastasis cascade may be acquired in certain tumors without the requirement that the associated cancer cells undergo additional mutations beyond those that were needed for primary tumor formation.

Plasticity in the Invasive Growth Program

The role of contextual signals in inducing an invasive growth capability (often via an EMT) implies the possibility of reversibility, in that cancer cells that have disseminated from a primary tumor to a more distant tissue site may no longer benefit from the activated stroma and invasion/EMT-inducing signals that they experienced while residing in the primary tumor; in the absence of ongoing exposure to these signals, carcinoma cells may revert in their new homes to a noninvasive state. Thus, carcinoma cells that have undergone an EMT during initial invasion and metastatic dissemination may pass through the reverse process, termed the mesenchymal-epithelial transition (MET). This plasticity may result in the formation of new tumor colonies of carcinoma cells exhibiting a histopathology similar to those of carcinoma cells in the primary tumor that never underwent an EMT (Hugo et al., 2007). Moreover, the notion that cancer cells routinely pass through a complete EMT program is likely to be

simplistic; instead, in many cases, cancer cells may enter into an EMT program only partially, thereby acquiring new mesenchymal traits while continuing to express residual epithelial traits.

Distinct Forms of Invasion May Underlie Different Cancer Types

The EMT program regulates a particular type of invasiveness that has been termed "mesenchymal." In addition, two other distinct modes of invasion have been identified and implicated in cancer cell invasion (Friedl and Wolf, 2008, 2010). "Collective invasion" involves nodules of cancer cells advancing en masse into adjacent tissues and is characteristic of, for example, squamous cell carcinomas; interestingly, such cancers are rarely metastatic, suggesting that this form of invasion lacks certain functional attributes that facilitate metastasis. Less clear is the prevalence of an "amoeboid" form of invasion (Madsen and Sahai, 2010; Sabeh et al., 2009), in which individual cancer cells show morphological plasticity, enabling them to slither through existing interstices in the extracellular matrix rather than clearing a path for themselves, as occurs in both the mesenchymal and collective forms of invasion. It is presently unresolved whether cancer cells participating in the collective and amoeboid forms of invasion employ components of the EMT program, or whether entirely different cell-biological programs are responsible for choreographing these alternative invasion programs.

Another emerging concept, noted above, involves the facilitation of cancer cell invasion by inflammatory cells that assemble at the boundaries of tumors, producing the extracellular matrix-degrading enzymes and other factors that enable invasive growth (Kessenbrock et al., 2010; Qian and Pollard, 2010; Joyce and Pollard, 2009); these functions may obviate the need of cancer cells to produce these proteins through activation of EMT programs. Thus, cancer cells may secrete the chemoattractants that recruit the proinvasive inflammatory cells rather than producing the matrix-degrading enzymes themselves.

The Daunting Complexity of Metastatic Colonization

Metastasis can be broken down into two major phases: the physical dissemination of cancer cells from the primary tumor to distant tissues, and the adaptation of these cells to foreign tissue microenvironments that results in successful colonization, i.e., the growth of micrometastases into macroscopic tumors. The multiple steps of dissemination would seem to be in the purview of the EMT and similarly acting migratory programs. Colonization, however, is not strictly coupled with physical dissemination, as evidenced by the presence in many patients of myriad micrometastases that have successfully disseminated but never progress to macroscopic metastatic tumors (Talmadge and Fidler, 2010; McGowan et al., 2009; Aguirre-Ghiso, 2007; Townson and Chambers, 2006; Fidler, 2003).

In some types of cancer, the primary tumor may release systemic suppressor factors that render such micrometastases dormant, as revealed clinically by explosive metastatic growth soon after resection of the primary growth (Demicheli et al., 2008; Folkman, 2002). In others, however, such as breast cancer and melanoma, macroscopic metastases may erupt decades after a primary tumor has been surgically removed or pharmacologically destroyed; these metastatic tumor growths evidently

Cell

reflect dormant micrometastases that have solved, after much trial and error, the complex problem of tissue colonization (Barkan, et al., 2010; Aguirre-Ghiso, 2007; Townson and Chambers, 2006).

One can infer from such natural histories that micrometastases may lack other hallmark capabilities necessary for vigorous growth, such as the ability to activate angiogenesis; indeed the inability of certain experimentally generated dormant micrometastases to form macroscopic tumors has been ascribed to their failure to activate tumor angiogenesis (Naumov et al., 2008; Aguirre-Ghiso, 2007). Additionally, recent experiments have shown that nutrient starvation can induce intense autophagy that causes cancer cells to shrink and adopt a state of reversible dormancy; such cells may exit this state and resume active growth and proliferation when changes in tissue microenvironment, such as access to more nutrients, permit (Kenific et al., 2010; Lu et al., 2008). Other mechanisms of micrometastatic dormancy may involve anti-growth signals embedded in normal tissue extracellular matrix (Barkan et al., 2010) and tumor-suppressing actions of the immune system (Teng et al., 2008; Aguirre-Ghiso, 2007).

Most disseminated cancer cells are likely to be poorly adapted, at least initially, to the microenvironment of the tissue in which they have landed. Accordingly, each type of disseminated cancer cell may need to develop its own set of ad hoc solutions to the problem of thriving in the microenvironment of one or another foreign tissue (Gupta et al., 2005). These adaptations might require hundreds of distinct colonization programs, each dictated by the type of disseminating cancer cell and the nature of the tissue microenvironment in which colonization is proceeding. As further discussed below, however, certain tissue microenviroments may be preordained to be intrinsically hospitable to disseminated cancer cells (Peinado et al., 2011; Talmadge and Fidler, 2010).

Metastatic dissemination has long been depicted as the last step in multistep primary tumor progression, and indeed for many tumors that is likely the case, as illustrated by recent genome sequencing studies that present genetic evidence for clonal evolution of pancreatic ductal adenocarcinoma to metastasis (Campbell et al., 2010; Luebeck, 2010; Yachida et al., 2010). On the other hand, evidence has recently emerged indicating that cells can disseminate remarkably early, dispersing from ostensibly noninvasive premalignant lesions in both mice and humans (Coghlin and Murray, 2010; Klein, 2009). Additionally, micrometastases can be spawned from primary tumors that are not obviously invasive but possess a neovasculature lacking in lumenal integrity (Gerhardt and Semb, 2008). Although cancer cells can clearly disseminate from such pre-neoplastic lesions and seed the bone marrow and other tissues, their capability to colonize these sites and develop into pathologically significant macrometastases remains unproven. At present, we view this early metastatic dissemination as a demonstrable phenomenon in mice and humans whose clinical significance is yet to be established.

Beyond the timing of their dissemination, it also remains unclear when and where cancer cells develop the ability to colonize foreign tissues as macroscopic tumors. This capability may arise during primary tumor formation as a result of a tumor's particular developmental path prior to any dissemination, such

that primary tumor cells entering the circulation are fortuitously endowed with the ability to colonize certain distant tissue sites (Talmadge and Fidler, 2010). Alternatively, the ability to colonize specific tissues may only develop in response to the selective pressure on already disseminated cancer cells to adapt to growth in foreign tissue microenvironments.

Having developed such tissue-specific colonizing ability, the cells in metastatic colonies may proceed to disseminate further, not only to new sites in the body but also back to the primary tumors in which their ancestors arose. Accordingly, tissue-specific colonization programs that are evident among cells within a primary tumor may originate not from classical tumor progression occurring within the primary lesion but instead from emigrants that have returned home (Kim et al., 2009). Such reseeding is consistent with the aforementioned studies of human pancreatic cancer metastasis (Campbell et al., 2010; Luebeck, 2010; Yachida et al., 2010). Stated differently, the phenotypes and underlying gene expression programs of the populations of cancer cells (and of the cancer stem cells discussed below) within primary tumors may be significantly modified by reverse migration of their distant metastatic progeny.

Implicit in this self-seeding process is another notion: the supportive stroma that arises in a primary tumor and contributes to its acquisition of malignant traits may intrinsically provide a hospitable site for reseeding and colonization by circulating cancer cells emanating from metastatic lesions.

Clarifying the regulatory programs that enable metastatic colonization represents an important agenda for future research. Substantial progress is being made, for example, in defining sets of genes ("metastatic signatures") that correlate with and appear to facilitate the establishment of macroscopic metastases in specific tissues (Coghlin and Murray, 2010; Bos et al., 2009; Olson et al., 2009; Nguyen et al., 2009; Gupta et al., 2005). The challenge is considerable, given the apparent multitude of distinct colonization programs cited above. Moreover, colonization is unlikely to depend exclusively on cell-autonomous processes. Instead, it almost certainly requires the establishment of a permissive tumor microenvironment composed of critical stromal support cells. For these reasons, the process of colonization is likely to encompass a large number of cell-biological programs that are, in aggregate, considerably more complex and diverse than the preceding steps of metastatic dissemination.

Programming of Hallmark Capabilities by Intracellular Circuitry

In 2000, we presented a metaphor, in which the numerous signaling molecules affecting cancer cells operate as nodes and branches of elaborate integrated circuits that are reprogrammed derivatives of the circuits operating in normal cells. The ensuing decade has both solidified the original depiction of these circuits and expanded the catalog of signals and the interconnections of their signaling pathways. It is difficult if not impossible to graphically portray this circuit comprehensively and coherently, as was already the case in 2000.

We now suggest a portrayal of this circuitry that is aligned with individual hallmarks of cancer. Thus, the intracellular integrated
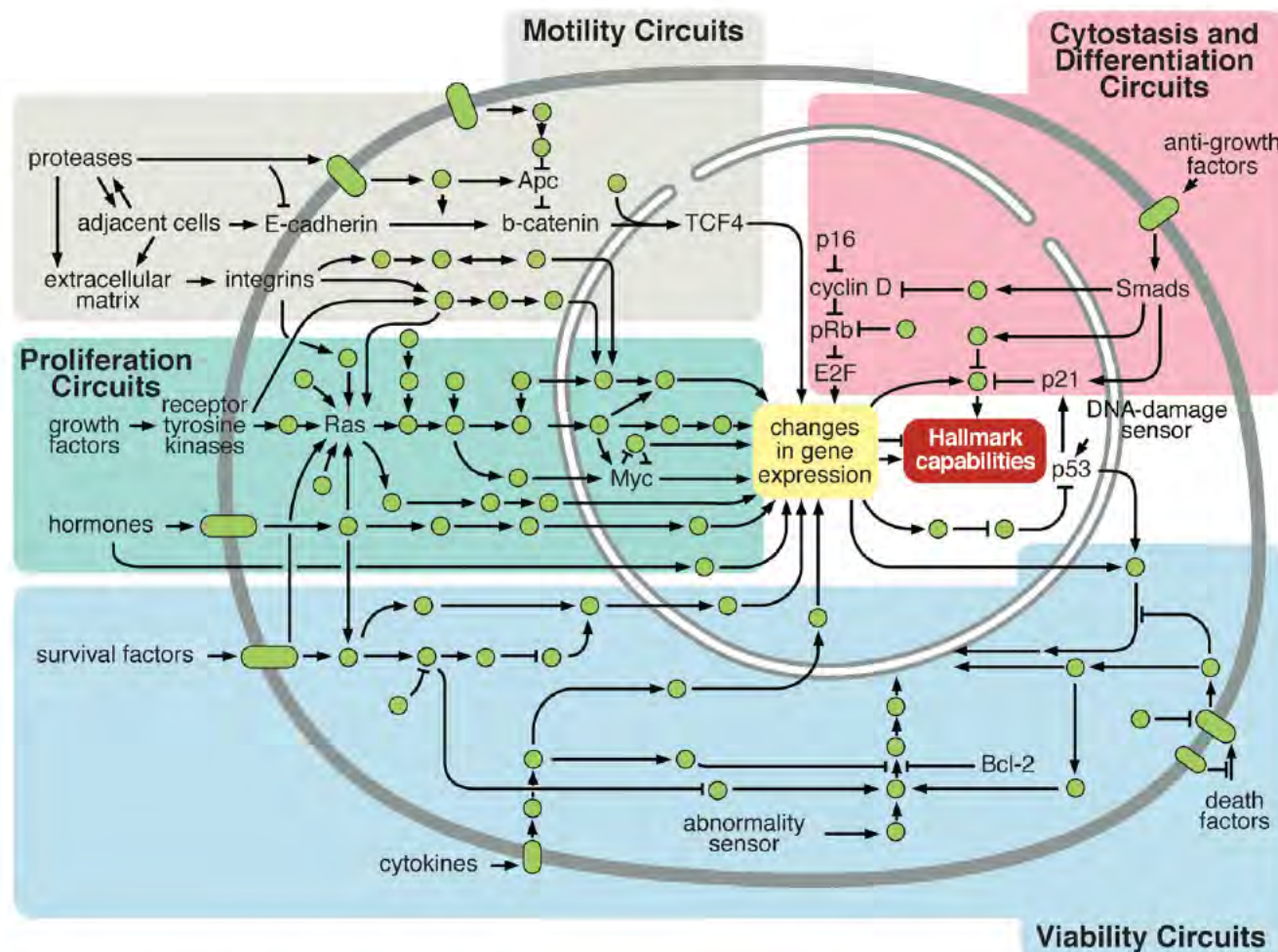
Figure 2. Intracellular Signaling Networks Regulate the Operations of the Cancer Cell
An elaborate integrated circuit operates within normal cells and is reprogrammed to regulate hallmark capabilities within cancer cells. Separate subcircuits, depicted here in differently colored fields, are specialized to orchestrate the various capabilities. At one level, this depiction is simplistic, as there is considerable crosstalk between such subcircuits. In addition, because each cancer cell is exposed to a complex mixture of signals from its microenvironment, each of these subcircuits is connected with signals originating from other cells in the tumor microenvironment, as outlined in Figure 5.

circuit can be segmented into distinct subcircuits, each of which is specialized to support a discrete cell-biological property in normal cells and is reprogrammed in order to implement a hallmark capability in cancer cells (Figure 2). Only a subset of hallmark capabilities are addressed in this figure, either because their underlying control circuits remain poorly understood or because they overlap extensively with those portrayed here.

An additional dimension of complexity involves considerable interconnections and thus crosstalk between the individual subcircuits. For example, certain oncogenic events can affect multiple capabilities, as illustrated by the diverse effects that prominent oncogenes, such as mutant *RAS* and upregulated *MYC*, have on multiple hallmark capabilities (e.g., proliferative signaling, energy metabolism, angiogenesis, invasion, and survival). We anticipate that future renditions of this integrated circuit will encompass subcircuits and associated hallmark capabilities that are still not addressed here.

ENABLING CHARACTERISTICS AND EMERGING HALLMARKS

We have defined the hallmarks of cancer as acquired functional capabilities that allow cancer cells to survive, proliferate, and disseminate; these functions are acquired in different tumor types via distinct mechanisms and at various times during the course of multistep tumorigenesis. Their acquisition is made possible by two *enabling characteristics*. Most prominent is the development of genomic instability in cancer cells, which generates random mutations including chromosomal rearrangements; among these are the rare genetic changes that can orchestrate hallmark capabilities. A second enabling characteristic involves the inflammatory state of premalignant and frankly malignant lesions that is driven by cells of the immune system, some of which serve to promote tumor progression through various means.

Cell
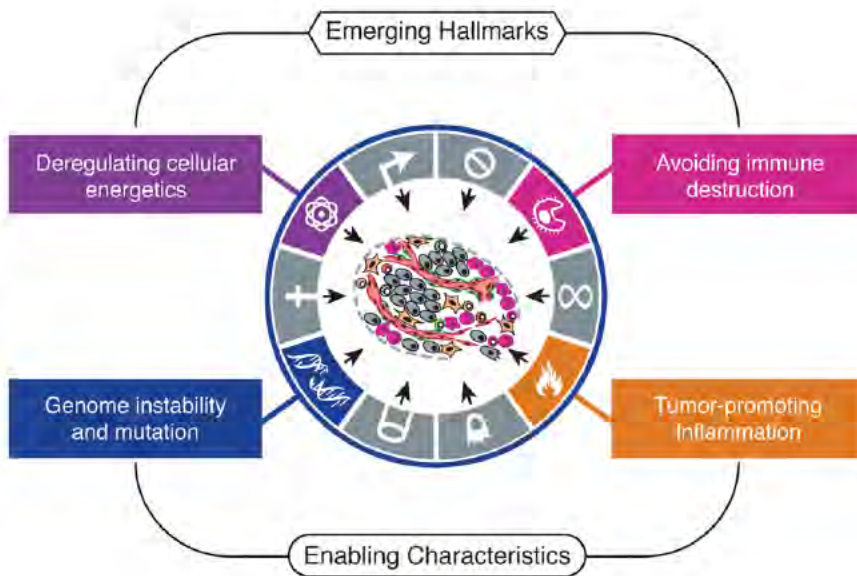
Figure 3. Emerging Hallmarks and Enabling Characteristics
An increasing body of research suggests that two additional hallmarks of cancer are involved in the pathogenesis of some and perhaps all cancers. One involves the capability to modify, or reprogram, cellular metabolism in order to most effectively support neoplastic proliferation. The second allows cancer cells to evade immunological destruction, in particular by T and B lymphocytes, macrophages, and natural killer cells. Because neither capability is yet generalized and fully validated, they are labeled as emerging hallmarks. Additionally, two consequential characteristics of neoplasia facilitate acquisition of both core and emerging hallmarks. Genomic instability and thus mutability endow cancer cells with genetic alterations that drive tumor progression. Inflammation by innate immune cells designed to fight infections and heal wounds can instead result in their inadvertent support of multiple hallmark capabilities, thereby manifesting the now widely appreciated tumor promoting consequences of inflammatory responses.

Yet other distinct attributes of cancer cells have been proposed to be functionally important for the development of cancer and might therefore be added to the list of core hallmarks (Negrini et al., 2010; Luo et al., 2009; Colotta et al., 2009). Two such attributes are particularly compelling. The first involves major reprogramming of cellular energy metabolism in order to support continuous cell growth and proliferation, replacing the metabolic program that operates in most normal tissues and fuels the physiological operations of the associated cells. The second involves active evasion by cancer cells from attack and elimination by immune cells; this capability highlights the dichotomous roles of an immune system that both antagonizes and enhances tumor development and progression. Both of these capabilities may well prove to facilitate the development and progression of many forms of human cancer and therefore can be considered to be emerging hallmarks of cancer. These enabling characteristics and *emerging hallmarks,* depicted in Figure 3, are discussed individually below.

An Enabling Characteristic: Genome Instability and Mutation

Acquisition of the multiple hallmarks enumerated above depends in large part on a succession of alterations in the genomes of neoplastic cells. Simply depicted, certain mutant genotypes confer selective advantage on subclones of cells, enabling their outgrowth and eventual dominance in a local tissue environment. Accordingly, multistep tumor progression can be portrayed as a succession of clonal expansions, each of which is triggered by the chance acquisition of an enabling mutant genotype. Because heritable phenotypes, e.g., inactivation of tumor suppressor genes, can also be acquired through epigenetic mechanisms such as DNA methylation and histone modifications (Berdasco and Esteller, 2010; Esteller, 2007; Jones and Baylin, 2007), some clonal expansions may well be triggered by nonmutational changes affecting the regulation of gene expression.

The extraordinary ability of genome maintenance systems to detect and resolve defects in the DNA ensures that rates of spontaneous mutation are usually very low during each cell generation. In the course of acquiring the roster of mutant genes needed to orchestrate tumorigenesis, cancer cells often increase the rates of mutation (Negrini et al., 2010; Salk et al., 2010). This mutability is achieved through increased sensitivity to mutagenic agents, through a breakdown in one or several components of the genomic maintenance machinery, or both. In addition, the accumulation of mutations can be accelerated by compromising the surveillance systems that normally monitor genomic integrity and force genetically damaged cells into either senescence or apoptosis (Jackson and Bartek, 2009; Kastan, 2008; Sigal and Rotter, 2000). The role of TP53 is central here, leading to its being called the "guardian of the genome" (Lane, 1992).

A diverse array of defects affecting various components of the DNA-maintenance machinery—often referred to as the "caretakers" of the genome (Kinzler and Vogelstein, 1997)—have been documented. The catalog of defects in these caretaker genes includes those whose products are involved in (1) detecting DNA damage and activating the repair machinery, (2) directly repairing damaged DNA, and (3) inactivating or intercepting mutagenic molecules before they have damaged the DNA (Negrini et al., 2010; Ciccia and Elledge, 2010; Jackson and Bartek, 2009; Kastan, 2008; Harper and Elledge, 2007; Friedberg et al., 2006). From a genetic perspective, these caretaker genes behave much like tumor suppressor genes, in that their functions can be lost during the course of tumor progression, with such losses being achieved either through inactivating mutations or via epigenetic repression. Mutant copies of many of these caretaker genes have been introduced into the mouse germline and result, predictably, in increased cancer incidence, supporting their potential involvement in human cancer development (Barnes and Lindahl, 2004).

Cell

In the decade since we first enumerated the cancer hallmarks, another major source of tumor-associated genomic instability has been uncovered: as described earlier, the loss of telomeric DNA in many tumors generates karyotypic instability and associated amplification and deletion of chromosomal segments (Artandi and DePinho, 2010). When viewed in this light, telomerase is more than an enabler of the hallmark capability for unlimited replicative potential and must also be added to the list of critical caretakers responsible for maintaining genome integrity.

Advances in the molecular-genetic analysis of cancer cell genomes have provided the most compelling demonstrations of function-altering mutations and of ongoing genomic instability during tumor progression. One type of analysis—comparative genomic hybridization (CGH)—documents the gains and losses of gene copy number across the cell genome; in many tumors, the pervasive genomic aberrations revealed by CGH provide clear evidence for loss of control of genome integrity. Importantly, the recurrence of specific aberrations (both amplifications and deletions) at particular sites in the genome indicates that such sites are likely to harbor genes whose alteration favors neoplastic progression (Korkola and Gray, 2010).

More recently, with the advent of efficient and economical DNA-sequencing technologies, higher-resolution analyses have become possible. Early studies are revealing distinctive patterns of DNA mutations in different tumor types (see http://cancergenome.nih.gov/). In the not-too-distant future, the sequencing of entire cancer cell genomes promises to clarify the prevalence of ostensibly random mutations scattered across cancer cell genomes. Thus, recurring genetic alterations may point to a causal role of particular mutations in tumor pathogenesis.

Although the specifics of genome alteration vary dramatically between different tumor types, the large number of genome maintenance and repair defects that have already been documented in human tumors, together with abundant evidence of widespread destabilization of gene copy number and nucleotide sequence, persuade us that instability of the genome is inherent to the great majority of human cancer cells. This leads, in turn, to the conclusion that the defects in genome maintenance and repair are selectively advantageous and therefore instrumental for tumor progression, if only because they accelerate the rate at which evolving premalignant cells can accumulate favorable genotypes. As such, genome instability is clearly an enabling characteristic that is causally associated with the acquisition of hallmark capabilities.

An Enabling Characteristic: Tumor-Promoting Inflammation

Pathologists have long recognized that some tumors are densely infiltrated by cells of both the innate and adaptive arms of the immune system and thereby mirror inflammatory conditions arising in non-neoplastic tissues (Dvorak, 1986). With the advent of better markers for accurately identifying the distinct cell types of the immune system, it is now clear that virtually every neoplastic lesion contains immune cells present at densities ranging from subtle infiltrations detectable only with cell type-specific antibodies to gross inflammations that are apparent even by standard histochemical staining techniques (Pagès et al., 2010). Historically, such immune responses were largely thought to reflect an attempt by the immune system to eradicate tumors, and indeed, there is increasing evidence for antitumoral responses to many tumor types with an attendant pressure on the tumor to evade immune destruction, as discussed below.

By 2000, there were already clues that the tumor-associated inflammatory response had the unanticipated, paradoxical effect of enhancing tumorigenesis and progression, in effect helping incipient neoplasias to acquire hallmark capabilities. In the ensuing decade, research on the intersections between inflammation and cancer pathogenesis has blossomed, producing abundant and compelling demonstrations of the functionally important tumor-promoting effects that immune cells—largely of the innate immune system—have on neoplastic progression (DeNardo et al., 2010; Grivennikov et al., 2010; Qian and Pollard, 2010; Colotta et al., 2009). Inflammation can contribute to multiple hallmark capabilities by supplying bioactive molecules to the tumor microenvironment, including growth factors that sustain proliferative signaling, survival factors that limit cell death, proangiogenic factors, extracellular matrix-modifying enzymes that facilitate angiogenesis, invasion, and metastasis, and inductive signals that lead to activation of EMT and other hallmark-facilitating programs (DeNardo et al., 2010; Grivennikov et al., 2010; Qian and Pollard, 2010; Karnoub and Weinberg, 2006–2007).

Importantly, inflammation is in some cases evident at the earliest stages of neoplastic progression and is demonstrably capable of fostering the development of incipient neoplasias into full-blown cancers (Qian and Pollard, 2010; de Visser et al., 2006). Additionally, inflammatory cells can release chemicals, notably reactive oxygen species, that are actively mutagenic for nearby cancer cells, accelerating their genetic evolution toward states of heightened malignancy (Grivennikov et al., 2010). As such, inflammation can be considered an enabling characteristic for its contributions to the acquisition of core hallmark capabilities. The cells responsible for this enabling characteristic are described in the section below on the tumor microenvironment.

An Emerging Hallmark: Reprogramming Energy Metabolism

The chronic and often uncontrolled cell proliferation that represents the essence of neoplastic disease involves not only deregulated control of cell proliferation but also corresponding adjustments of energy metabolism in order to fuel cell growth and division. Under aerobic conditions, normal cells process glucose, first to pyruvate via glycolysis in the cytosol and thereafter to carbon dioxide in the mitochondria; under anaerobic conditions, glycolysis is favored and relatively little pyruvate is dispatched to the oxygen-consuming mitochondria. Otto Warburg first observed an anomalous characteristic of cancer cell energy metabolism (Warburg, 1930, 1956a, 1956b): even in the presence of oxygen, cancer cells can reprogram their glucose metabolism, and thus their energy production, by limiting their energy metabolism largely to glycolysis, leading to a state that has been termed "aerobic glycolysis."

The existence of this metabolic switch in cancer cells has been substantiated in the ensuing decades. Such reprogramming of

Cell

energy metabolism is seemingly counterintuitive, in that cancer cells must compensate for the ~18-fold lower efficiency of ATP production afforded by glycolysis relative to mitochondrial oxidative phosphorylation. They do so in part by upregulating glucose transporters, notably GLUT1, which substantially increases glucose import into the cytoplasm (Jones and Thompson, 2009; DeBerardinis et al., 2008; Hsu and Sabatini, 2008). Indeed, markedly increased uptake and utilization of glucose have been documented in many human tumor types, most readily by noninvasively visualizing glucose uptake using positron emission tomography (PET) with a radiolabeled analog of glucose (^{18}F-fluorodeoxyglucose, FDG) as a reporter.

Glycolytic fueling has been shown to be associated with activated oncogenes (e.g., *RAS, MYC*) and mutant tumor suppressors (e.g., *TP53*) (DeBerardinis et al., 2008; Jones and Thompson, 2009), whose alterations in tumor cells have been selected primarily for their benefits in conferring the hallmark capabilities of cell proliferation, avoidance of cytostatic controls, and attenuation of apoptosis. This reliance on glycolysis can be further accentuated under the hypoxic conditions that operate within many tumors: the hypoxia response system acts pleiotropically to upregulate glucose transporters and multiple enzymes of the glycolytic pathway (Semenza, 2010a; Jones and Thompson, 2009; DeBerardinis et al., 2008). Thus, both the Ras oncoprotein and hypoxia can independently increase the levels of the HIF1α and HIF2α transcription factors, which in turn upregulate glycolysis (Semenza, 2010a, 2010b; Kroemer and Pouyssegur, 2008).

A functional rationale for the glycolytic switch in cancer cells has been elusive, given the relatively poor efficiency of generating ATP by glycolysis relative to mitochondrial oxidative phosphorylation. According to one long-forgotten (Potter, 1958) and recently revived and refined hypothesis (Vander Heiden et al., 2009), increased glycolysis allows the diversion of glycolytic intermediates into various biosynthetic pathways, including those generating nucleosides and amino acids; this facilitates, in turn, the biosynthesis of the macromolecules and organelles required for assembling new cells. Moreover, Warburg-like metabolism seems to be present in many rapidly dividing embryonic tissues, once again suggesting a role in supporting the large-scale biosynthetic programs that are required for active cell proliferation.

Interestingly, some tumors have been found to contain two subpopulations of cancer cells that differ in their energy-generating pathways. One subpopulation consists of glucose-dependent ("Warburg-effect") cells that secrete lactate, whereas cells of the second subpopulation preferentially import and utilize the lactate produced by their neighbors as their main energy source, employing part of the citric acid cycle to do so (Kennedy and Dewhirst, 2010; Feron, 2009; Semenza, 2008). These two populations evidently function symbiotically: the hypoxic cancer cells depend on glucose for fuel and secrete lactate as waste, which is imported and preferentially used as fuel by their better-oxygenated brethren. Although this provocative mode of intratumoral symbiosis has yet to be generalized, the cooperation between lactate-secreting and lactate-utilizing cells to fuel tumor growth is in fact not an invention of tumors but rather again reflects cooption of a normal physiological mechanism, in this case one operating in

muscle (Kennedy and Dewhirst, 2010; Feron, 2009; Semenza, 2008). Additionally, it is becoming apparent that oxygenation, ranging from normoxia to hypoxia, is not necessarily static in tumors but instead fluctuates temporally and regionally (Hardee et al., 2009), likely as a result of the instability and chaotic organization of the tumor-associated neovasculature.

Altered energy metabolism is proving to be as widespread in cancer cells as many of the other cancer-associated traits that have been accepted as hallmarks of cancer. This realization raises the question of whether deregulating cellular energy metabolism is therefore a core hallmark capability of cancer cells that is as fundamental as the six well-established core hallmarks. In fact, the redirection of energy metabolism is largely orchestrated by proteins that are involved in one way or another in programming the core hallmarks of cancer. When viewed in this way, aerobic glycolysis is simply another phenotype that is programmed by proliferation-inducing oncogenes.

Interestingly, activating (gain-of-function) mutations in the isocitrate dehydrogenase 1/2 (IDH) enzymes have been reported in glioma and other human tumors (Yen et al., 2010). Although these mutations may prove to have been clonally selected for their ability to alter energy metabolism, there is confounding data associating their activity with elevated oxidation and stability of the HIF-1 transcription factors (Reitman and Yan, 2010), which could in turn affect genome stability and angiogenesis/invasion, respectively, thus blurring the lines of phenotypic demarcation. Currently, therefore, the designation of reprogrammed energy metabolism as an emerging hallmark seems most appropriate, to highlight both its evident importance as well as the unresolved issues surrounding its functional independence from the core hallmarks.

An Emerging Hallmark: Evading Immune Destruction
A second, still-unresolved issue surrounding tumor formation involves the role that the immune system plays in resisting or eradicating formation and progression of incipient neoplasias, late-stage tumors, and micrometastases. The long-standing theory of immune surveillance proposes that cells and tissues are constantly monitored by an ever-alert immune system, and that such immune surveillance is responsible for recognizing and eliminating the vast majority of incipient cancer cells and thus nascent tumors. According to this logic, solid tumors that do appear have somehow managed to avoid detection by the various arms of the immune system or have been able to limit the extent of immunological killing, thereby evading eradication.

The role of defective immunological monitoring of tumors would seem to be validated by the striking increases of certain cancers in immunocompromised individuals (Vajdic and van Leeuwen, 2009). However, the great majority of these are virus-induced cancers, suggesting that much of the control of this class of cancers normally depends on reducing viral burden in infected individuals, in part through eliminating virus-infected cells. These observations, therefore, seem to shed little light on the possible role of the immune system in limiting formation of the >80% of tumors of nonviral etiology. In recent years, however, an increasing body of evidence, both from genetically engineered mice and from clinical epidemiology, suggests that

Cell

the immune system operates as a significant barrier to tumor formation and progression, at least in some forms of non-virus-induced cancer.

When mice genetically engineered to be deficient for various components of the immune system were assessed for the development of carcinogen-induced tumors, it was observed that tumors arose more frequently and/or grew more rapidly in the immunodeficient mice relative to immunocompetent controls. In particular, deficiencies in the development or function of $CD8^+$ cytotoxic T lymphocytes (CTLs), $CD4^+$ T_h1 helper T cells, or natural killer (NK) cells each led to demonstrable increases in tumor incidence; moreover, mice with combined immunodeficiencies in both T cells and NK cells were even more susceptible to cancer development. The results indicated that, at least in certain experimental models, both the innate and adaptive cellular arms of the immune system are able to contribute significantly to immune surveillance and thus tumor eradication (Teng et al., 2008; Kim et al., 2007).

In addition, transplantation experiments have shown that cancer cells that originally arose in immunodeficient mice are often inefficient at initiating secondary tumors in syngeneic immunocompetent hosts, whereas cancer cells from tumors arising in immunocompetent mice are equally efficient at initiating transplanted tumors in both types of hosts (Teng et al., 2008; Kim et al., 2007). Such behavior has been interpreted as follows: Highly immunogenic cancer cell clones are routinely eliminated in immunocompetent hosts—a process that has been referred to as "immunoediting"—leaving behind only weakly immunogenic variants to grow and generate solid tumors; such weakly immunogenic cells can thereafter colonize both immunodeficient and immunocompetent hosts. Conversely, when arising in immunodeficient hosts, the immunogenic cancer cells are not selectively depleted and can, instead, prosper along with their weakly immunogenic counterparts. When cells from such nonedited tumors are serially transplanted into syngeneic recipients, the immunogenic cancer cells are rejected when they confront, for the first time, the competent immune systems of their secondary hosts (Smyth et al., 2006). (Unanswered in these particular experiments is the question of whether the chemical carcinogens used to induce such tumors are prone to generate cancer cells that are especially immunogenic.)

Clinical epidemiology also increasingly supports the existence of antitumoral immune responses in some forms of human cancer (Bindea et al., 2010; Ferrone and Dranoff, 2010; Nelson, 2008). For example, patients with colon and ovarian tumors that are heavily infiltrated with CTLs and NK cells have a better prognosis than those that lack such abundant killer lymphocytes (Pagès et al., 2010; Nelson, 2008); the case for other cancers is suggestive but less compelling and is the subject of ongoing investigation. Additionally, some immunosuppressed organ transplant recipients have been observed to develop donor-derived cancers, suggesting that in the ostensibly tumor-free donors, the cancer cells were held in check, in a dormant state, by a fully functional immune system (Strauss and Thomas, 2010).

Still, the epidemiology of chronically immunosuppressed patients does not indicate significantly increased incidences of the major forms of nonviral human cancer, as noted above.

This might be taken as an argument against the importance of immune surveillance as an effective barrier to tumorigenesis and tumor progression. We note, however, that HIV and pharmacologically immunosuppressed patients are predominantly immunodeficient in the T and B cell compartments and thus do not present with the multicomponent immunological deficiencies that have been produced in the genetically engineered mutant mice lacking both NK cells and CTLs; this leaves open the possibility that such patients still have residual capability for an immunological defense against cancer that is mounted by NK and other innate immune cells.

In truth, the above discussions of cancer immunology simplify tumor-host immunological interactions, as highly immunogenic cancer cells may well evade immune destruction by disabling components of the immune system that have been dispatched to eliminate them. For example, cancer cells may paralyze infiltrating CTLs and NK cells, by secreting TGF-β or other immunosuppressive factors (Yang et al., 2010; Shields et al., 2010). More subtle mechanisms operate through the recruitment of inflammatory cells that are actively immunosuppressive, including regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs). Both can suppress the actions of cytotoxic lymphocytes (Mougiakakos et al., 2010; Ostrand-Rosenberg and Sinha, 2009).

In light of these considerations and the still-rudimentary demonstrations of antitumor immunity as a significant barrier to tumor formation and progression in humans, we present immunoevasion as another emerging hallmark, whose generality as a core hallmark capability remains to be firmly established.

THE TUMOR MICROENVIRONMENT

Over the past decade, tumors have increasingly been recognized as organs whose complexity approaches and may even exceed that of normal healthy tissues. When viewed from this perspective, the biology of a tumor can only be understood by studying the individual specialized cell types within it (Figure 4, upper) as well as the "tumor microenvironment" that they construct during the course of multistep tumorigenesis (Figure 4, lower). This depiction contrasts starkly with the earlier, reductionist view of a tumor as nothing more than a collection of relatively homogeneous cancer cells, whose entire biology could be understood by elucidating the cell-autonomous properties of these cells. We enumerate here a set of cell types known to contribute in important ways to the biology of many tumors and discuss the regulatory signaling that controls their individual and collective functions. Most of these observations stem from the study of carcinomas, in which the neoplastic epithelial cells constitute a compartment (the parenchyma) that is clearly distinct from the mesenchymal cells forming the tumor-associated stroma.

Cancer Cells and Cancer Stem Cells

Cancer cells are the foundation of the disease; they initiate tumors and drive tumor progression forward, carrying the oncogenic and tumor suppressor mutations that define cancer as a genetic disease. Traditionally, the cancer cells within tumors

often-rare tumor-initiating cells proved to share transcriptional profiles with certain normal tissue stem cell populations, motivating their designation as stem-like.

The origins of CSCs within a solid tumor have not been clarified and indeed may well vary from one tumor type to another. In some tumors, normal tissue stem cells may serve as the cells-of-origin that undergo oncogenic transformation to yield CSCs; in others, partially differentiated transit-amplifying cells, also termed progenitor cells, may suffer the initial oncogenic transformation thereafter assuming more stem-like character. Once primary tumors have formed, the CSCs, like their normal counterparts, may self-renew as well as spawn more differentiated derivatives; in the case of neoplastic CSCs, these descendant cells form the great bulk of many tumors. It remains to be established whether multiple distinct classes of increasingly neoplastic stem cells form during inception and subsequent multistep progression of tumors, ultimately yielding the CSCs that have been described in fully developed cancers.

Recent research has interrelated the acquisition of CSC traits with the EMT transdifferentiation program discussed above (Singh and Settleman, 2010; Mani et al., 2008; Morel et al., 2008). Induction of this program in certain model systems can induce many of the defining features of stem cells, including self-renewal ability and the antigenic phenotypes associated with both normal and cancer stem cells. This concordance suggests that the EMT program not only may enable cancer cells to physically disseminate from primary tumors but also can confer on such cells the self-renewal capability that is crucial to their subsequent clonal expansion at sites of dissemination (Brabletz et al., 2005). If generalized, this connection raises an important corollary hypothesis: the heterotypic signals that trigger an EMT, such as those released by an activated, inflammatory stroma, may also be important in creating and maintaining CSCs.

have been portrayed as reasonably homogeneous cell populations until relatively late in the course of tumor progression, when hyperproliferation combined with increased genetic instability spawn distinct clonal subpopulations. Reflecting such clonal heterogeneity, many human tumors are histopathologically diverse, containing regions demarcated by various degrees of differentiation, proliferation, vascularity, inflammation, and/or invasiveness. In recent years, however, evidence has accumulated pointing to the existence of a new dimension of intratumor heterogeneity and a hitherto-unappreciated subclass of neoplastic cells within tumors, termed cancer stem cells (CSCs).

Although the evidence is still fragmentary, CSCs may prove to be a common constituent of many if not most tumors, albeit being present with widely varying abundance. CSCs are defined operationally through their ability to efficiently seed new tumors upon inoculation into recipient host mice (Cho and Clarke, 2008; Lobo et al., 2007). This functional definition is often complemented by including the expression in CSCs of markers that are also expressed by the normal stem cells in the tissue-of-origin (Al-Hajj et al., 2003).

CSCs were initially implicated in the pathogenesis of hematopoietic malignancies (Reya et al., 2001; Bonnet and Dick, 1997) and then years later were identified in solid tumors, in particular breast carcinomas and neuroectodermal tumors (Gilbertson and Rich, 2007; Al-Hajj et al., 2003). Fractionation of cancer cells on the basis of displayed cell-surface markers has yielded subpopulations of neoplastic cells with a greatly enhanced ability, relative to the corresponding majority populations, to seed new tumors upon implantation in immunodeficient mice. These

An increasing number of human tumors are reported to contain subpopulations with the properties of CSCs, as defined operationally through their efficient tumor-initiating capabilities upon xenotransplantation into mice. Nevertheless, the importance of CSCs as a distinct phenotypic subclass of neoplastic cells remains a matter of debate, as does their oft-cited rarity within tumors (Boiko et al., 2010; Gupta et al., 2009; Quintana et al., 2008). Indeed, it is plausible that the phenotypic plasticity operating within tumors may produce bidirectional interconversion between CSCs and non-CSCs, resulting in dynamic variation in the relative abundance of CSCs. Such plasticity could complicate definitive measurement of their prevalence. Analogous plasticity is already implicated in the EMT program, which can be engaged reversibly (Thiery and Sleeman, 2006).

These complexities notwithstanding, it is evident that this new dimension of tumor heterogeneity holds important implications for successful cancer therapies. Increasing evidence in a variety of tumor types suggests that cells with properties of CSCs are more resistant to various commonly used chemotherapeutic treatments (Singh and Settleman, 2010; Creighton et al., 2009; Buck et al., 2007). Their persistence may help to explain the almost-inevitable disease recurrence following apparently successful debulking of human solid tumors by radiation and various forms of chemotherapy. Indeed, CSCs may well prove to underlie certain forms of tumor dormancy, whereby latent cancer cells persist for years or even decades after surgical resection or radio/chemotherapy, only to suddenly erupt and generate life-threatening disease. Hence, CSCs may represent a double-threat, in that they are more resistant to therapeutic killing and, at the same time, endowed with the ability to regenerate a tumor once therapy has been halted.

This phenotypic plasticity implicit in CSC state may also enable the formation of functionally distinct subpopulations within a tumor that support overall tumor growth in various ways. For example, an EMT can convert epithelial carcinoma cells into mesenchymal, fibroblast-like cancer cells that may well assume the duties of cancer-associated fibroblasts (CAFs) in some tumors. Remarkably, several recent reports have documented the ability of glioblastoma cells (or possibly their associated CSC subpopulations) to transdifferentiate into endothelial-like cells that can substitute for bona fide host-derived endothelial cells in forming a tumor-associated neovasculature (Soda et al., 2011; El Hallani et al., 2010; Ricci-Vitiani et al., 2010; Wang et al., 2010). Observations like these indicate that certain tumors may acquire stromal support by inducing some of their own cancer cells to undergo various types of metamorphosis to produce stromal cell types rather than relying on recruited host cells to provide their functions.

The discovery of CSCs and biological plasticity in tumors indicates that a single, genetically homogeneous population of cells within a tumor may nevertheless be phenotypically heterogeneous due to the presence of cells in distinct states of differentiation. However, an equally important source of phenotypic variability may derive from the genetic heterogeneity within a tumor that accumulates as cancer progression proceeds. Thus, elevated genetic instability operating in later stages of

tumor progression may drive rampant genetic diversification that outpaces the process of Darwinian selection, generating genetically distinct subpopulations far more rapidly than they can be eliminated.

Such thinking is increasingly supported by in-depth sequence analysis of tumor cell genomes, which has become practical due to recent major advances in DNA (and RNA) sequencing technology. Thus the sequencing of the genomes of cancer cells microdissected from different sectors of the same tumor (Yachida et al., 2010) has revealed striking intratumoral genetic heterogeneity. Some of this genetic diversity may be reflected in the long-recognized histological heterogeneity within individual human tumors. Alternatively, this genetic diversification may enable functional specialization, producing subpopulations of cancer cells that contribute distinct, complementary capabilities, which then accrue to the common benefit of overall tumor growth as described above.

Endothelial Cells

Much of the cellular heterogeneity within tumors is found in their stromal compartments. Prominent among the stromal constituents are the cells forming the tumor-associated vasculature. Mechanisms of development, differentiation, and homeostasis of endothelial cells composing the arteries, veins, and capillaries were already well understood in 2000. So too was the concept of the "angiogenic switch," which activates quiescent endothelial cells, causing them to enter into a cell-biological program that allows them to construct new blood vessels (see above). Over the last decade, a network of interconnected signaling pathways involving ligands of signal-transducing receptors displayed by endothelial cells (e.g., Notch, Neuropilin, Robo, and Eph-A/B) has been added to the already-prominent VEGF, angiopoietin, and FGF signals. These newly characterized pathways have been functionally implicated in developmental and tumor-associated angiogenesis and illustrate the complex regulation of endothelial cell phenotypes (Pasquale, 2010; Ahmed and Bicknell, 2009; Dejana et al., 2009; Carmeliet and Jain, 2000).

Other avenues of research are revealing distinctive gene expression profiles of tumor-associated endothelial cells and identifying cell-surface markers displayed on the lumenal surfaces of normal versus tumor endothelial cells (Nagy et al., 2010; Ruoslahti et al., 2010; Ruoslahti, 2002). Differences in signaling, in transcriptome profiles, and in vascular "ZIP codes" will likely prove to be important for understanding the conversion of normal endothelial cells into tumor-associated endothelial cells. Such knowledge may lead, in turn, to opportunities to develop novel therapies that exploit these differences in order to selectively target tumor-associated endothelial cells.

Closely related to the endothelial cells of the general circulation are those forming lymphatic vessels (Tammela and Alitalo, 2010). Their role in the tumor-associated stroma, specifically in supporting tumor growth, is poorly understood. Indeed, because of high interstitial pressure within solid tumors, intratumoral lymphatic vessels are typically collapsed and nonfunctional; in contrast, however, there are often functional, actively growing ("lymphangiogenic") lymphatic vessels at the peripheries of tumors and in the adjacent normal tissues that cancer cells

Cell

invade. These associated lymphatics likely serve as channels for the seeding of metastases in the draining lymph nodes that are commonly observed in a number of cancer types.

Pericytes

As noted earlier, pericytes represent a specialized mesenchymal cell type (related to smooth muscle cells) with finger-like projections that wrap around the endothelial tubing of blood vessels. In normal tissues, pericytes are known to provide paracrine support signals to the normally quiescent endothelium. For example, Ang-1 secreted by pericytes conveys antiproliferative stabilizing signals that are received by the Tie2 receptors expressed on the surface of endothelial cells; some pericytes also produce low levels of VEGF that serve a trophic function in endothelial homeostasis (Gaengel et al., 2009; Bergers and Song, 2005). Pericytes also collaborate with the endothelial cells to synthesize the vascular basement membrane that anchors both pericytes and endothelial cells and helps vessel walls to withstand the hydrostatic pressure of blood flow.

Genetic and pharmacological perturbation of the recruitment and association of pericytes has demonstrated the functional importance of these cells in supporting the tumor endothelium (Pietras and Ostman, 2010; Gaengel et al., 2009; Bergers and Song, 2005). For example, pharmacological inhibition of signaling through the PDGF receptor expressed by tumor pericytes and bone marrow-derived pericyte progenitors results in reduced pericyte coverage of tumor vessels, which in turn destabilizes vascular integrity and function (Pietras and Ostman, 2010; Raza et al., 2010; Gaengel et al., 2009); interestingly, and in contrast, the pericytes of normal vessels are not prone to such pharmacological disruption, providing another example of the differences in regulation of normal quiescent and tumor vasculature. An intriguing hypothesis, still to be fully substantiated, is that tumors with poor pericyte coverage of their vasculature may be more prone to permit cancer cell intravasation into the circulatory system, enabling subsequent hematogenous dissemination (Raza et al., 2010; Gerhardt and Semb, 2008).

Immune Inflammatory Cells

As also discussed above, infiltrating cells of the immune system are increasingly accepted to be generic constituents of tumors. These inflammatory cells operate in conflicting ways: both tumor-antagonizing and tumor-promoting leukocytes can be found, in various proportions, in most if not all neoplastic lesions. Although the presence of tumor-antagonizing CTLs and NK cells is not surprising, the prevalence of immune cells that functionally enhance hallmark capabilities was largely unanticipated. Evidence began to accumulate in the late 1990s that the infiltration of neoplastic tissues by cells of the immune system serves, perhaps counterintuitively, to promote tumor progression. Such work traced its conceptual roots back to the association of sites of chronic inflammation with tumor formation, and to the observation that tumors could be portrayed as wounds that never heal (Schäfer and Werner, 2008: Dvorak, 1986). In the course of normal wound healing and fighting infections, immune inflammatory cells appear transiently and then disappear, in contrast to their persistence in sites of chronic inflammation, where their presence has been associated with various tissue pathologies,

including fibrosis, aberrant angiogenesis, and neoplasia (Grivennikov et al., 2010; Karin et al., 2006).

Over the past decade, the manipulation of genes involved in the determination or effector functions of various immune cell types, together with pharmacological inhibitors of such cells or their functions, has shown them to play diverse and critical roles in fostering tumorigenesis. The roster of tumor-promoting inflammatory cells now includes macrophage subtypes, mast cells, and neutrophils, as well as T and B lymphocytes (Coffelt et al., 2010; DeNardo et al., 2010; Egeblad et al., 2010; Johansson et al., 2008; Murdoch et al., 2008; DePalma et al., 2007). Such studies are yielding a growing list of signaling molecules released by inflammatory cells that serve as effectors of their tumor-promoting actions. These include the tumor growth factor EGF, the angiogenic growth factor VEGF, other proangiogenic factors such as FGF2, chemokines, and cytokines that amplify the inflammatory state; in addition, these cells may produce proangiogenic and/or proinvasive matrix-degrading enzymes, including MMP-9 and other matrix metalloproteinases, cysteine cathepsin proteases, and heparanase (Qian and Pollard, 2010; Murdoch et al., 2008). Consistent with their expression of these diverse effectors, tumor-infiltrating inflammatory cells have been shown to induce and help sustain tumor angiogenesis, to stimulate cancer cell proliferation, to facilitate, via their presence at the margins of tumors, tissue invasion, and to support the metastatic dissemination and seeding of cancer cells (Coffelt et al., 2010; Egeblad et al., 2010; Qian and Pollard, 2010; Mantovani, 2010; Joyce and Pollard, 2009; Mantovani et al., 2008; Murdoch et al., 2008; DePalma et al., 2007).

In addition to fully differentiated immune cells present in tumor stroma, a variety of partially differentiated myeloid progenitors have been identified in tumors (Murdoch et al., 2008). Such cells represent intermediaries between circulating cells of bone marrow origin and the differentiated immune cells typically found in normal and inflamed tissues. Importantly, these progenitors, like their more differentiated derivatives, have demonstrable tumor-promoting activity. Of particular interest, a class of tumor-infiltrating myeloid cells (defined as coexpressing the macrophage marker CD11b and the neutrophil marker Gr1) has been shown to suppress CTL and NK cell activity, having been independently identified as MDSCs (Qian and Pollard, 2010; Ostrand-Rosenberg and Sinha, 2009). This attribute raises the possibility that recruitment of certain myeloid cells may be doubly beneficial for the developing tumor, by directly promoting angiogenesis and tumor progression while at the same time affording a means to evade immune destruction.

The counterintuitive existence of both tumor-promoting and tumor-antagonizing immune cells can be rationalized by invoking the diverse roles of the immune system: On the one hand, the immune system specifically detects and targets infectious agents with the adaptive immune response, which is supported by cells of the innate immune system. On the other, the innate immune system is involved in wound healing and clearing dead cells and cellular debris. These specialized tasks are accomplished by distinct subclasses of inflammatory cells, namely a class of conventional macrophages and neutrophils (engaged in supporting adaptive immunity), and subclasses of "alternatively activated" macrophages, neutrophils, and

Cell

myeloid progenitors that are engaged in wound healing and tissue housecleaning (Egeblad et al., 2010; Mantovani, 2010; Qian and Pollard, 2010; Johansson et al., 2008). The latter subtypes of immune cells are one of the major sources of the angiogenic, epithelial, and stromal growth factors and matrix-remodeling enzymes that are needed for wound healing, and it is these cells that are recruited and subverted to support neoplastic progression. Similarly, subclasses of B and T lymphocytes may facilitate the recruitment, activation, and persistence of such wound-healing and tumor-promoting macrophages and neutrophils (DeNardo et al., 2010; Egeblad et al., 2010; Biswas and Mantovani, 2010). Of course, other subclasses of B and T lymphocytes and innate immune cell types can mount demonstrable tumor-killing responses. The balance between the conflicting inflammatory responses in tumors is likely to prove instrumental in prognosis and, quite possibly, in therapies designed to redirect these cells toward tumor destruction.

Cancer-Associated Fibroblasts

Fibroblasts are found in various proportions across the spectrum of carcinomas, constituting in many cases the preponderant cell population of the tumor stroma. The term "cancer-associated fibroblast" subsumes at least two distinct cell types: (1) cells with similarities to the fibroblasts that create the structural foundation supporting most normal epithelial tissues and (2) myofibroblasts, whose biological roles and properties differ markedly from those of tissue-derived fibroblasts. Myofibroblasts are identifiable by their expression of α-smooth muscle actin (SMA). They are rare in most healthy epithelial tissues, although certain tissues, such as the liver and pancreas, contain appreciable numbers of α-SMA-expressing cells. Myofibroblasts transiently increase in abundance in wounds and are also found in sites of chronic inflammation. Although beneficial to tissue repair, myofibroblasts are problematic in chronic inflammation, contributing to the pathological fibrosis observed in tissues such as lung, kidney, and liver.

Recruited myofibroblasts and reprogrammed variants of normal tissue-derived fibroblastic cells have been demonstrated to enhance tumor phenotypes, notably cancer cell proliferation, angiogenesis, and invasion and metastasis; their tumor-promoting activities have largely been defined by transplantation of cancer-associated fibroblasts admixed with cancer cells into mice, and more recently by genetic and pharmacologic perturbation of their functions in tumor-prone mice (Dirat et al., 2010; Pietras and Ostman, 2010; Räsänen and Vaheri, 2010; Shimoda et al., 2010; Kalluri and Zeisberg, 2006; Bhowmick et al., 2004). Because they secrete a variety of extracellular matrix components, cancer-associated fibroblasts are implicated in the formation of the desmoplastic stroma that characterizes many advanced carcinomas. The full spectrum of functions contributed by both subtypes of cancer-associated fibroblasts to tumor pathogenesis remains to be elucidated.

Stem and Progenitor Cells of the Tumor Stroma

The various stromal cell types that constitute the tumor microenvironment may be recruited from adjacent normal tissue—the most obvious reservoir of such cell types. However, in recent years, the bone marrow has increasingly been implicated as a key source of tumor-associated stromal cells (Bergfeld and DeClerck, 2010; Fang and Salven, 2011; Giaccia and Schipani, 2010; Patenaude et al., 2010; Lamagna and Bergers, 2006). Mesenchymal stem and progenitor cells have been found to transit into tumors from the marrow, where they may differentiate into the various well-characterized stromal cell types. Some of these recent arrivals may also persist in an undifferentiated or partially differentiated state, exhibiting functions that their more differentiated progeny lack.

The bone marrow origins of stromal cell types have been demonstrated using tumor-bearing mice in which the bone marrow cells and thus their disseminated progeny have been selectively labeled with reporters such as green fluorescent protein (GFP). While immune inflammatory cells have been long known to derive from the bone marrow, more recently the progenitors of pericytes and of various subtypes of cancer-associated fibroblasts originating from the bone marrow have been described in various mouse models of cancer (Bergfeld and DeClerck, 2010; Fang and Salven, 2011; Giaccia and Schipani, 2010; Lamagna and Bergers, 2006); the prevalence and functional importance of endothelial progenitors for tumor angiogenesis is currently unresolved (Fang and Salven, 2011; Patenaude et al., 2010). Taken together, these various lines of evidence indicate that tumor-associated stromal cells may be supplied to growing tumors by proliferation of preexisting stromal cells, by differentiation in situ of local stem/progenitor cells originating in the neighboring normal tissue, or via recruitment of bone marrow-derived stem/progenitor cells.

Heterotypic Signaling Orchestrates the Cells of the Tumor Microenvironment

Depictions of the intracellular circuitry governing cancer cell biology (e.g., Figure 2) will need to be complemented by similar diagrams charting the complex interactions between the neoplastic and stromal cells within a tumor and the dynamic extracellular matrix that they collectively erect and remodel (Egeblad et al., 2010; Kessenbrock et al., 2010; Pietras and Ostman, 2010; Polyak et al., 2009). A reasonably complete, graphic depiction of the network of microenvironmental signaling interactions is still far beyond our reach, as the great majority of signaling molecules and pathways remain to be identified. We provide instead a hint of such interactions in Figure 5, upper. These few well-established examples are intended to exemplify a signaling network of remarkable complexity that is of critical importance to tumor pathogenesis.

Another dimension of complexity is not represented in this simple schematic: both neoplastic cells and the stromal cells around them change progressively during the multistep transformation of normal tissues into high-grade malignancies. This histopathological progression must reflect underlying changes in heterotypic signaling between tumor parenchyma and stroma.

Such stepwise progression is likely to depend on back-and-forth reciprocal interactions between the neoplastic cells and the supporting stromal cells, as depicted in Figure 5, lower. Thus, incipient neoplasias begin the interplay by recruiting and activating stromal cell types that assemble into an initial preneoplastic stroma, which in turn responds reciprocally by enhancing
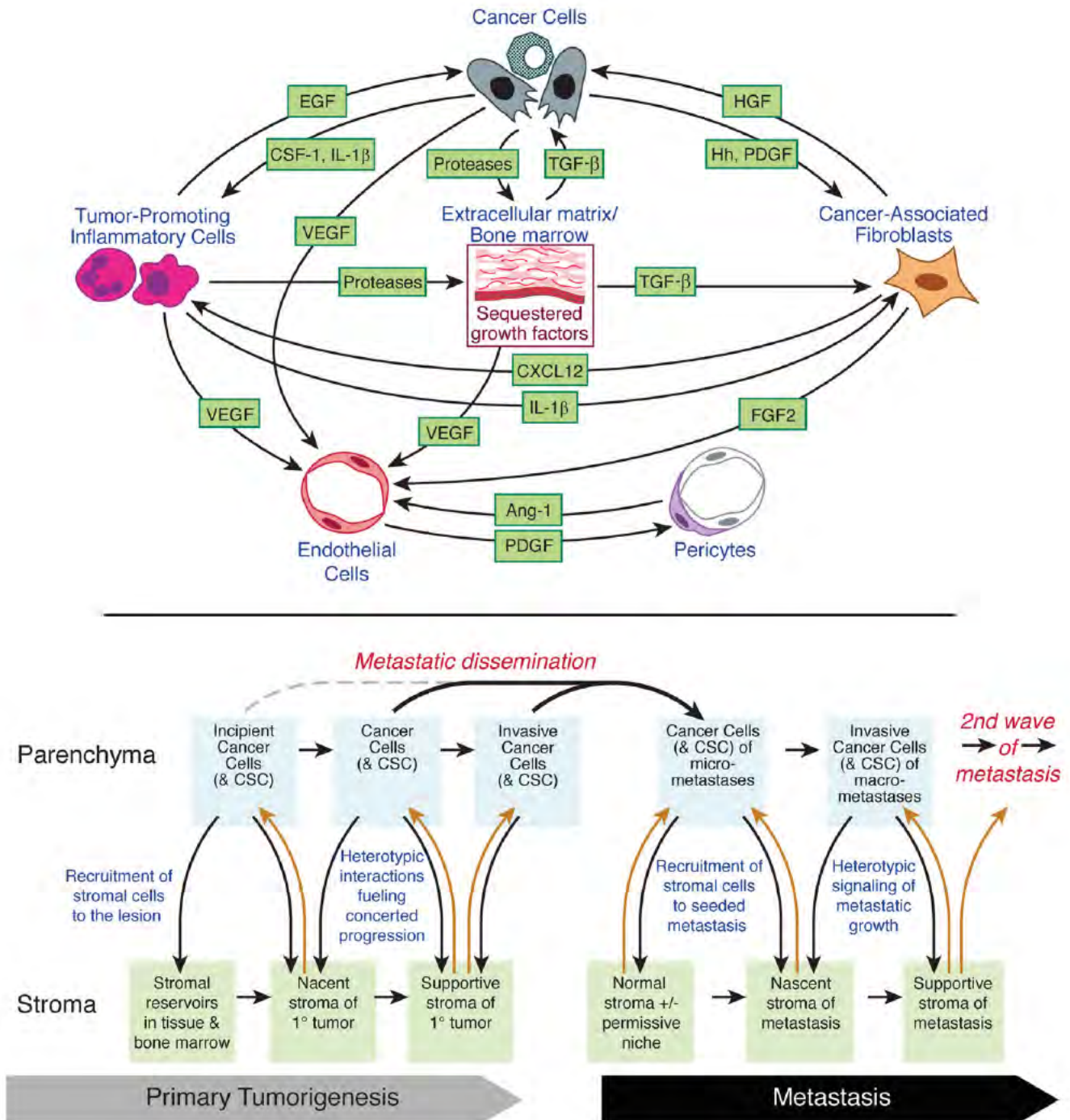
Cell

Figure 5. Signaling Interactions in the Tumor Microenvironment during Malignant Progression

(Upper) The assembly and collective contributions of the assorted cell types constituting the tumor microenvironment are orchestrated and maintained by reciprocal heterotypic signaling interactions, of which only a few are illustrated.

(Lower) The intracellular signaling depicted in the upper panel within the tumor microenvironment is not static but instead changes during tumor progression as a result of reciprocal signaling interactions between cancer cells of the parenchyma and stromal cells that convey the increasingly aggressive phenotypes that underlie growth, invasion, and metastatic dissemination. Importantly, the predisposition to spawn metastatic lesions can begin early, being influenced by the differentiation program of the normal cell of origin or by initiating oncogenic lesions. Certain organ sites (sometimes referred to as "fertile soil" or "metastatic niches") can be especially permissive for metastatic seeding and colonization by certain types of cancer cells, as a consequence of local properties that are either intrinsic to the normal tissue or induced at a distance by systemic actions of primary tumors. Cancer stem cells may be variably involved in some or all of the different stages of primary tumorigenesis and metastasis.

the neoplastic phenotypes of the nearby cancer cells. The cancer cells, which may further evolve genetically, again feed signals back to the stroma, continuing the reprogramming of normal stromal cells to serve the budding neoplasm; ultimately signals originating in the tumor stroma enable cancer cells to invade normal adjacent tissues and disseminate.

This model of reciprocal heterotypic signaling must be extended to encompass the final stage of multistep tumor progression—metastasis (Figure 5, lower right). The circulating cancer cells that are released from primary tumors leave a micro-environment created by the supportive stroma of such tumors. However, upon landing in a distant organ, these cancer cells encounter a naive, fully normal, tissue microenvironment. Consequently, many of the heterotypic signals that shaped their phenotype while they resided within primary tumors may be absent in sites of dissemination, constituting a barrier to growth of the seeded cancer cells. Thus, the succession of reciprocal cancer cell to stromal cell interactions that defined multistep progression in the primary tumor now must be repeated anew in distant tissues as disseminated cancer cells proceed to colo-nize their newfound organ sites.

Although this logic applies in some cases of metastasis, in others, as mentioned earlier, certain tissue microenvironments may, for various reasons, already be supportive of freshly seeded cancer cells; such permissive sites have been referred to as "metastatic niches" (Peinado et al., 2011; Coghlin and Murray, 2010). Implicit in this term is the notion that cancer cells seeded in such sites may not need to begin by inducing a supportive stroma because it already preexists, at least in part. Such permissivity may be intrinsic to the tissue site (Talmadge and Fidler, 2010) or preinduced by circulating factors released by the primary tumor (Peinado et al., 2011). The most well-documented components of induced premetastatic niches are tumor-promoting inflammatory cells, although other cell types and the ECM may well prove to play important roles in different metastatic contexts.

The likelihood that signaling interactions between cancer cells and their supporting stroma evolve during the course of multi-stage tumor development clearly complicates the goal of fully elucidating the mechanisms of cancer pathogenesis. For example, this reality poses challenges to systems biologists seeking to chart the crucial regulatory networks than orchestrate malignant progression. Moreover, it seems likely that under-standing these dynamic variations will become crucial to the development of novel therapies designed to successfully target both primary and metastatic tumors.

THERAPEUTIC TARGETING

The introduction of mechanism-based targeted therapies to treat human cancers has been heralded as one of the fruits of three decades of remarkable progress of research into the mechanisms of cancer pathogenesis. We do not attempt here to enumerate the myriad therapies that are under development or have been introduced of late into the clinic. Instead, we consider how the description of hallmark principles is beginning to inform therapeutic development at present and may increas-ingly do so in the future.

The rapidly growing armamentarium of targeted therapeutics can be categorized according to their respective effects on one or more hallmark capabilities, as illustrated in the examples presented in Figure 6. Indeed, the observed efficacy of these drugs represents, in each case, a validation of a particular capa-bility: if a capability is truly important for the biology of tumors, then its inhibition should impair tumor growth and progression.

We note that most of the hallmark-targeting cancer drugs developed to date have been deliberately directed toward specific molecular targets that are involved in one way or another in enabling particular capabilities. Such specificity of action has been considered a virtue, as it presents inhibitory activity against a target while having, in principle, relatively fewer off-target effects and thus less nonspecific toxicity. In fact, resulting clin-ical responses have generally been transitory, being followed by almost-inevitable relapses.

One interpretation of this history, supported by growing exper-imental evidence, is that each of the core hallmark capabilities is regulated by partially redundant signaling pathways. Conse-quently, a targeted therapeutic agent inhibiting one key pathway in a tumor may not completely shut off a hallmark capability, al-lowing some cancer cells to survive with residual function until they or their progeny eventually adapt to the selective pressure imposed by the therapy being applied. Such adaptation, which can be accomplished by mutation, epigenetic reprogramming, or remodeling of the stromal microenvironment, can reestablish the functional capability, permitting renewed tumor growth and clinical relapse. Given that the number of parallel signaling path-ways supporting a given hallmark must be limited, it may become possible to target all of these supporting pathways ther-apeutically, thereby preventing the development of adaptive resistance.

In response to therapy, cancer cells may also reduce their dependence on a particular hallmark capability, becoming more dependent on another; this represents a quite different form of acquired drug resistance. This concept is exemplified by recent discoveries of unexpected responses to antiangio-genic therapies. Some have anticipated that effective inhibition of angiogenesis would render tumors dormant and might even lead to their dissolution (Folkman and Kalluri, 2004). Instead, the clinical responses to antiangiogenic therapies have been found to be transitory (Azam et al., 2010; Ebos et al., 2009; Berg-ers and Hanahan, 2008).

In certain preclinical models, where potent angiogenesis inhib-itors succeed in suppressing this hallmark capability, tumors adapt and shift from a dependence upon continuing angiogen-esis to heightening the activity of another instead—invasiveness and metastasis (Azam et al., 2010: Ebos et al., 2009; Bergers and Hanahan, 2008). By invading nearby tissues, initially hypoxic cancer cells evidently gain access to normal, preexisting tissue vasculature. Initial clinical validation of this adaptive/evasive resistance is apparent in the increased invasion and local metas-tasis seen when human glioblastomas are treated with antian-giogenic therapies (Ellis and Reardon, 2009; Norden et al., 2009; Verhoeff et al., 2009). The applicability of this lesson to other human cancers has yet to be established.

Analogous adaptive shifts in dependence on other hallmark traits may also limit efficacy of analogous hallmark-targeting
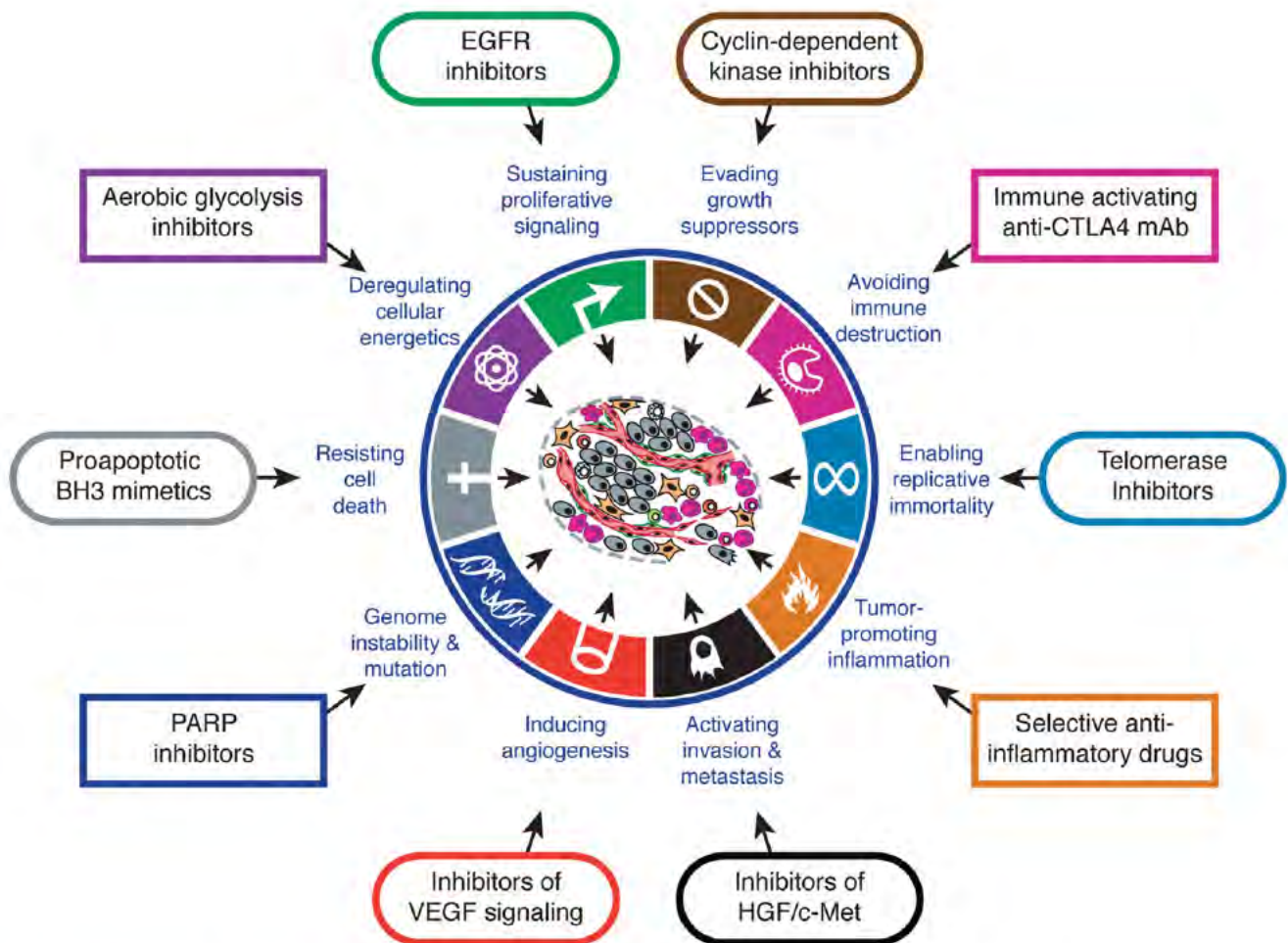
Figure 6. Therapeutic Targeting of the Hallmarks of Cancer

Drugs that interfere with each of the acquired capabilities necessary for tumor growth and progression have been developed and are in clinical trials or in some cases approved for clinical use in treating certain forms of human cancer. Additionally, the investigational drugs are being developed to target each of the enabling characteristics and emerging hallmarks depicted in Figure 3, which also hold promise as cancer therapeutics. The drugs listed are but illustrative examples; there is a deep pipeline of candidate drugs with different molecular targets and modes of action in development for most of these hallmarks.

therapies. For example, the deployment of apoptosis-inducing drugs may induce cancer cells to hyperactivate mitogenic signaling, enabling them to compensate for the initial attrition triggered by such treatments. Such considerations suggest that drug development and the design of treatment protocols will benefit from incorporating the concepts of functionally discrete hallmark capabilities and of the multiple biochemical pathways involved in supporting each of them. Thus, in particular, we can envisage that selective cotargeting of multiple core and emerging hallmark capabilities and enabling characteristics (Figure 6) in mechanism-guided combinations will result in more effective and durable therapies for human cancer.

CONCLUSION AND FUTURE VISION

We have sought here to revisit, refine, and extend the concept of cancer hallmarks, which has provided a useful conceptual framework for understanding the complex biology of cancer.

The six acquired capabilities—the hallmarks of cancer—have stood the test of time as being integral components of most forms of cancer. Further refinement of these organizing principles will surely come in the foreseeable future, continuing the remarkable conceptual progress of the last decade.

Looking ahead, we envision significant advances during the coming decade in our understanding of invasion and metastasis. Similarly, the role of aerobic glycolysis in malignant growth will be elucidated, including a resolution of whether this metabolic reprogramming is a discrete capability separable from the core hallmark of chronically sustained proliferation. We remain perplexed as to whether immune surveillance is a barrier that virtually all tumors must circumvent, or only an idiosyncrasy of an especially immunogenic subset of them; this issue too will be resolved in one way or another.

Yet other areas are currently in rapid flux. In recent years, elaborate molecular mechanisms controlling transcription through chromatin modifications have been uncovered, and there are

Cell

clues that specific shifts in chromatin configuration occur during the acquisition of certain hallmark capabilities (Berdasco and Esteller, 2010). Functionally significant epigenetic alterations seem likely to be factors not only in the cancer cells but also in the altered cells of the tumor-associated stroma. It is unclear at present whether an elucidation of these epigenetic mechanisms will materially change our overall understanding of the means by which hallmark capabilities are acquired or simply add additional detail to the regulatory circuitry that is already known to govern them.

Similarly, the discovery of hundreds of distinct regulatory microRNAs has already led to profound changes in our understanding of the genetic control mechanisms that operate in health and disease. By now dozens of microRNAs have been implicated in various tumor phenotypes (Garzon et al., 2010), and yet these only scratch the surface of the real complexity, as the functions of hundreds of microRNAs known to be present in our cells and altered in expression in different forms of cancer remain total mysteries. Here again, we are unclear as to whether future progress will cause fundamental shifts in our understanding of the pathogenetic mechanisms of cancer or only add detail to the elaborate regulatory circuits that have already been mapped out.

Finally, the circuit diagrams of heterotypic interactions between the multiple distinct cell types that assemble and collaborate to produce different forms and progressively malignant stages of cancer are currently rudimentary. In another decade, we anticipate that the signaling circuitry describing the intercommunication between these various cells within tumors will be charted in far greater detail and clarity, eclipsing our current knowledge. And, as before (Hanahan and Weinberg, 2000), we continue to foresee cancer research as an increasingly logical science, in which myriad phenotypic complexities are manifestations of a small set of underlying organizing principles.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures that are downloadable for presentations and can be found with this article online at doi:10.1016/j.cell.2011.02.013.

REFERENCES

Adams, J.M., and Cory, S. (2007). The Bcl 2 apoptotic switch in cancer development and therapy. Oncogene 26, 1324 1337.

Aguirre Ghiso, J.A. (2007). Models, mechanisms and clinical evidence for cancer dormancy. Nat. Rev. Cancer 7, 834 846.

Ahmed, Z., and Bicknell, R. (2009). Angiogenic signalling pathways. Methods Mol. Biol. 467, 3 24.

Al Hajj, M., Wicha, M.S., Benito Hernandez, A., Morrison, S.J., and Clarke, M.F. (2003). Prospective identification of tumorigenic breast cancer cells. Proc. Natl. Acad. Sci. USA 100, 3983 3988.

Amaravadi, R.K., and Thompson, C.B. (2007). The roles of therapy induced autophagy and necrosis in cancer treatment. Clin. Cancer Res. 13, 7271 7279.

Amit, I., Citri, A., Shay, T., Lu, Y., Katz, M., Zhang, F., Tarcic, G., Siwak, D., La had, J., Jacob Hirsch, J., et al. (2007). A module of negative feedback regulators defines growth factor signaling. Nat. Genet. 39, 503 512.

Apel, A., Zentgraf, H., Büchler, M.W., and Herr, I. (2009). Autophagy A double edged sword in oncology. Int. J. Cancer 125, 991 995.

Artandi, S.E., and DePinho, R.A. (2000). Mice without telomerase: what can they teach us about human cancer? Nat. Med. 6, 852 855.

Artandi, S.E., and DePinho, R.A. (2010). Telomeres and telomerase in cancer. Carcinogenesis 31, 9 18.

Azam, F., Mehta, S., and Harris, A.L. (2010). Mechanisms of resistance to anti angiogenesis therapy. Eur. J. Cancer 46, 1323 1332.

Baeriswyl, V., and Christofori, G. (2009). The angiogenic switch in carcinogenesis. Semin. Cancer Biol. 19, 329 337.

Baluk, P., Hashizume, H., and McDonald, D.M. (2005). Cellular abnormalities of blood vessels as targets in cancer. Curr. Opin. Genet. Dev. 15, 102 111.

Barkan, D., Green, J.E., and Chambers, A.F. (2010). Extracellular matrix: a gatekeeper in the transition from dormancy to metastatic growth. Eur. J. Cancer 46, 1181 1188.

Barnes, D.E., and Lindahl, T. (2004). Repair and genetic consequences of endogenous DNA base damage in mammalian cells. Annu. Rev. Genet. 38, 445 476.

Barrallo Gimeno, A., and Nieto, M.A. (2005). The Snail genes as inducers of cell movement and survival: implications in development and cancer. Development 132, 3151 3161.

Berdasco, M., and Esteller, M. (2010). Aberrant epigenetic landscape in cancer: How cellular identity goes awry. Dev. Cell 19, 698 711.

Bergers, G., and Benjamin, L.E. (2003). Tumorigenesis and the angiogenic switch. Nat. Rev. Cancer 3, 401 410.

Bergers, G., and Hanahan, D. (2008). Modes of resistance to anti angiogenic therapy. Nat. Rev. Cancer 8, 592 603.

Bergers, G., and Song, S. (2005). The role of pericytes in blood vessel formation and maintenance. Neuro oncol. 7, 452 464.

Bergfeld, S.A., and DeClerck, Y.A. (2010). Bone marrow derived mesenchymal stem cells and the tumor microenvironment. Cancer Metastasis Rev. 29, 249 261.

Berx, G., and van Roy, F. (2009). Involvement of members of the cadherin superfamily in cancer. Cold Spring Harb. Perspect. Biol. 1, a003129.

Bhowmick, N.A., Neilson, E.G., and Moses, H.L. (2004). Stromal fibroblasts in cancer initiation and progression. Nature 432, 332 337.

Bierie, B., and Moses, H.L. (2006). Tumour microenvironment: TGFbeta: the molecular Jekyll and Hyde of cancer. Nat. Rev. Cancer 6, 506 520.

Bindea, G., Mlecnik, B., Fridman, W.H., Pagès, F., and Galon, J. (2010). Natural immunity to cancer in humans. Curr. Opin. Immunol. 22, 215 222.

Biswas, S.K., and Mantovani, A. (2010). Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. Nat. Immunol. 11, 889 896.

Blasco, M.A. (2005). Telomeres and human disease: ageing, cancer and beyond. Nat. Rev. Genet. 6, 611 622.

Boiko, A.D., Razorenova, O.V., van de Rijn, M., Swetter, S.M., Johnson, D.L., Ly, D.P., Butler, P.D., Yang, G.P., Joshua, B., Kaplan, M.J., et al. (2010). Human melanoma initiating cells express neural crest nerve growth factor receptor CD271. Nature 466, 133 137.

Bonnet, D., and Dick, J.E. (1997). Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat. Med. 3, 730 737.

Bos, P.D., Zhang, X.H., Nadal, C., Shu, W., Gomis, R.R., Nguyen, D.X., Minn, A.J., van de Vijver, M.J., Gerald, W.L., Foekens, J.A., and Massagué, J. (2009). Genes that mediate breast cancer metastasis to the brain. Nature 459, 1005 1009.

Cell

Brabletz, T., Jung, A., Reu, S., Porzner, M., Hlubek, F., Kunz Schughart, L.A., Knuechel, R., and Kirchner, T. (2001). Variable beta catenin expression in colorectal cancers indicates tumor progression driven by the tumor environment. Proc. Natl. Acad. Sci. USA 98, 10356 10361.

Brabletz, T., Jung, A., Spaderna, S., Hlubek, F., and Kirchner, T. (2005). Opinion: migrating cancer stem cells   an integrated concept of malignant tumour progression. Nat. Rev. Cancer 5, 744 749.

Buck, E., Eyzaguirre, A., Barr, S., Thompson, S., Sennello, R., Young, D., Iwata, K.K., Gibson, N.W., Cagnoni, P., and Haley, J.D. (2007). Loss of homo typic cell adhesion by epithelial mesenchymal transition or mutation limits sensitivity to epidermal growth factor receptor inhibition. Mol. Cancer Ther. 6, 532 541.

Burkhart, D.L., and Sage, J. (2008). Cellular mechanisms of tumour suppres sion by the retinoblastoma gene. Nat. Rev. Cancer 8, 671 682.

Cabrita, M.A., and Christofori, G. (2008). Sprouty proteins, masterminds of receptor tyrosine kinase signaling. Angiogenesis 11, 53 62.

Campbell, P.J., Yachida, S., Mudie, L.J., Stephens, P.J., Pleasance, E.D., Stebbings, L.A., Morsberger, L.A., Latimer, C., McLaren, S., Lin, M.L., et al. (2010). The patterns and dynamics of genomic instability in metastatic pancre atic cancer. Nature 467, 1109 1113.

Cao, Y. (2010). Adipose tissue angiogenesis as a therapeutic target for obesity and metabolic diseases. Nat. Rev. Drug Discov. 9, 107 115.

Carmeliet, P. (2005). VEGF as a key mediator of angiogenesis in cancer. Oncology 69 (Suppl 3), 4 10.

Carmeliet, P., and Jain, R.K. (2000). Angiogenesis in cancer and other diseases. Nature 407, 249 257.

Cavallaro, U., and Christofori, G. (2004). Cell adhesion and signalling by cad herins and Ig CAMs in cancer. Nat. Rev. Cancer 4, 118 132.

Cheng, N., Chytil, A., Shyr, Y., Joly, A., and Moses, H.L. (2008). Transforming growth factor beta signaling deficient fibroblasts enhance hepatocyte growth factor signaling in mammary carcinoma cells to promote scattering and inva sion. Mol. Cancer Res. 6, 1521 1533.

Chin, K., de Solorzano, C.O., Knowles, D., Jones, A., Chou, W., Rodriguez, E.G., Kuo, W.L., Ljung, B.M., Chew, K., Myambo, K., et al. (2004). In situ anal yses of genome instability in breast cancer. Nat. Genet. 36, 984 988.

Cho, R.W., and Clarke, M.F. (2008). Recent advances in cancer stem cells. Curr. Opin. Genet. Dev. 18, 1 6.

Ciccia, A., and Elledge, S.J. (2010). The DNA damage response: making it safe to play with knives. Mol. Cell 40, 179 204.

Coffelt, S.B., Lewis, C.E., Naldini, L., Brown, J.M., Ferrara, N., and De Palma, M. (2010). Elusive identities and overlapping phenotypes of proangiogenic myeloid cells in tumors. Am. J. Pathol. 176, 1564 1576.

Coghlin, C., and Murray, G.I. (2010). Current and emerging concepts in tumour metastasis. J. Pathol. 222, 1 15.

Collado, M., and Serrano, M. (2010). Senescence in tumours: evidence from mice and humans. Nat. Rev. Cancer 10, 51 57.

Colotta, F., Allavena, P., Sica, A., Garlanda, C., and Mantovani, A. (2009). Cancer related inflammation, the seventh hallmark of cancer: links to genetic instability. Carcinogenesis 30, 1073 1081.

Cong, Y., and Shay, J.W. (2008). Actions of human telomerase beyond telo meres. Cell Res. 18, 725 732.

Creighton, C.J., Li, X., Landis, M., Dixon, J.M., Neumeister, V.M., Sjolund, A., Rimm, D.L., Wong, H., Rodriguez, A., Herschkowitz, J.I., et al. (2009). Residual breast cancers after conventional therapy display mesenchymal as well as tumor initiating features. Proc. Natl. Acad. Sci. USA 106, 13820 13825.

Curto, M., Cole, B.K., Lallemand, D., Liu, C.H., and McClatchey, A.I. (2007). Contact dependent inhibition of EGFR signaling by Nf2/Merlin. J. Cell Biol. 177, 893 903.

Davies, M.A., and Samuels, Y. (2010). Analysis of the genome to personalize therapy for melanoma. Oncogene 29, 5545 5555.

DeBerardinis, R.J., Lum, J.J., Hatzivassiliou, G., and Thompson, C.B. (2008). The biology of cancer: Metabolic reprogramming fuels cell growth and prolif eration. Cell Metab. 7, 11 20.

Dejana, E., Orsenigo, F., Molendini, C., Baluk, P., and McDonald, D.M. (2009). Organization and signaling of endothelial cell to cell junctions in various regions of the blood and lymphatic vascular trees. Cell Tissue Res. 335, 17 25.

Demicheli, R., Retsky, M.W., Hrushesky, W.J., Baum, M., and Gukas, I.D. (2008). The effects of surgery on tumor growth: a century of investigations. Ann. Oncol. 19, 1821 1828.

DeNardo, D.G., Andreu, P., and Coussens, L.M. (2010). Interactions between lymphocytes and myeloid cells regulate pro versus anti tumor immunity. Cancer Metastasis Rev. 29, 309 316.

De Palma, M., Murdoch, C., Venneri, M.A., Naldini, L., and Lewis, C.E. (2007). Tie2 expressing monocytes: regulation of tumor angiogenesis and therapeutic implications. Trends Immunol. 28, 519 524.

Deshpande, A., Sicinski, P., and Hinds, P.W. (2005). Cyclins and cdks in devel opment and cancer: a perspective. Oncogene 24, 2909 2915.

de Visser, K.E., Eichten, A., and Coussens, L.M. (2006). Paradoxical roles of the immune system during cancer development. Nat. Rev. Cancer 6, 24 37.

Dirat, B., Bochet, L., Escourrou, G., Valet, P., and Muller, C. (2010). Unraveling the obesity and breast cancer links: a role for cancer associated adipocytes? Endocr. Dev. 19, 45 52.

Dvorak, H.F. (1986). Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. N. Engl. J. Med. 315, 1650 1659.

Ebos, J.M., Lee, C.R., and Kerbel, R.S. (2009). Tumor and host mediated path ways of resistance and disease progression in response to antiangiogenic therapy. Clin. Cancer Res. 15, 5020 5025.

Egeblad, M., Nakasone, E.S., and Werb, Z. (2010). Tumors as organs: complex tissues that interface with the entire organism. Dev. Cell 18, 884 901.

El Hallani, S., Boisselier, B., Peglion, F., Rousseau, A., Colin, C., Idbaih, A., Marie, Y., Mokhtari, K., Thomas, J.L., Eichmann, A., et al. (2010). A new alter native mechanism in glioblastoma vascularization: tubular vasculogenic mimicry. Brain 133, 973 982.

Ellis, L.M., and Reardon, D.A. (2009). Cancer: The nuances of therapy. Nature 458, 290 292.

Esteller, M. (2007). Cancer epigenomics: DNA methylomes and histone modi fication maps. Nat. Rev. Genet. 8, 286 298.

Evan, G.I., and d'Adda di Fagagna, F. (2009). Cellular senescence: hot or what? Curr. Opin. Genet. Dev. 19, 25 31.

Evan, G., and Littlewood, T. (1998). A matter of life and cell death. Science 281, 1317 1322.

Fang, S., and Salven, P. (2011). Stem cells in tumor angiogenesis. J. Mol. Cell. Cardiol. 50, 290 295.

Feron, O. (2009). Pyruvate into lactate and back: from the Warburg effect to symbiotic energy fuel exchange in cancer cells. Radiother. Oncol. 92, 329 333.

Feldser, D.M., and Greider, C.W. (2007). Short telomeres limit tumor progres sion in vivo by inducing senescence. Cancer Cell 11, 461 469.

Ferrara, N. (2009). Vascular endothelial growth factor. Arterioscler. Thromb. Vasc. Biol. 29, 789 791.

Ferrara, N. (2010). Pathways mediating VEGF independent tumor angiogen esis. Cytokine Growth Factor Rev. 21, 21 26.

Ferrone, C., and Dranoff, G. (2010). Dual roles for immunity in gastrointestinal cancers. J. Clin. Oncol. 28, 4045 4051.

Fidler, I.J. (2003). The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited. Nat. Rev. Cancer 3, 453 458.

Folkman, J. (2002). Role of angiogenesis in tumor growth and metastasis. Semin. Oncol. 29(6, Suppl 16), 15 18.

Folkman, J. (2006). Angiogenesis. Annu. Rev. Med. 57, 1 18.

Folkman, J., and Kalluri, R. (2004). Cancer without disease. Nature 427, 787.

Cell

Friedberg, E.C., Aguilera, A., Gellert, M., Hanawalt, P.C., Hays, J.B., Lehmann, A.R., Lindahl, T., Lowndes, N., Sarasin, A., and Wood, R.D. (2006). DNA repair: from molecular mechanism to human disease. DNA Repair (Amst.) 5, 986 996.

Friedl, P., and Wolf, K. (2008). Tube travel: the role of proteases in individual and collective cancer cell invasion. Cancer Res. 68, 7247 7249.

Friedl, P., and Wolf, K. (2010). Plasticity of cell migration: a multiscale tuning model. J. Cell Biol. 188, 11 19.

Gaengel, K., Genové, G., Armulik, A., and Betsholtz, C. (2009). Endothelial mural cell signaling in vascular development and angiogenesis. Arterioscler. Thromb. Vasc. Biol. 29, 630 638.

Galluzzi, L., and Kroemer, G. (2008). Necroptosis: a specialized pathway of programmed necrosis. Cell 135, 1161 1163.

Garzon, R., Marcucci, G., and Croce, C.M. (2010). Targeting microRNAs in cancer: rationale, strategies and challenges. Nat. Rev. Drug Discov. 9, 775 789.

Gerhardt, H., and Semb, H. (2008). Pericytes: gatekeepers in tumour cell metastasis? J. Mol. Med. 86, 135 144.

Ghebranious, N., and Donehower, L.A. (1998). Mouse models in tumor suppression. Oncogene 17, 3385 3400.

Giaccia, A.J., and Schipani, E. (2010). Role of carcinoma associated fibroblasts and hypoxia in tumor progression. Curr. Top. Microbiol. Immunol. 345, 31 45.

Gilbertson, R.J., and Rich, J.N. (2007). Making a tumour's bed: glioblastoma stem cells and the vascular niche. Nat. Rev. Cancer 7, 733 736.

Gocheva, V., Wang, H.W., Gadea, B.B., Shree, T., Hunter, K.E., Garfall, A.L., Berman, T., and Joyce, J.A. (2010). IL 4 induces cathepsin protease activity in tumor associated macrophages to promote cancer growth and invasion. Genes Dev. 24, 241 255.

Grivennikov, S.I., Greten, F.R., and Karin, M. (2010). Immunity, inflammation, and cancer. Cell 140, 883 899.

Gupta, G.P., Minn, A.J., Kang, Y., Siegel, P.M., Serganova, I., Cordón Cardo, C., Olshen, A.B., Gerald, W.L., and Massagué, J. (2005). Identifying site specific metastasis genes and functions. Cold Spring Harb. Symp. Quant. Biol. 70, 149 158.

Gupta, P.B., Chaffer, C.L., and Weinberg, R.A. (2009). Cancer stem cells: mirage or reality? Nat. Med. 15, 1010 1012.

Hanahan, D., and Folkman, J. (1996). Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell 86, 353 364.

Hanahan, D., and Weinberg, R.A. (2000). The hallmarks of cancer. Cell 100, 57 70.

Hansel, D.E., Meeker, A.K., Hicks, J., De Marzo, A.M., Lillemoe, K.D., Schulick, R., Hruban, R.H., Maitra, A., and Argani, P. (2006). Telomere length variation in biliary tract metaplasia, dysplasia, and carcinoma. Mod. Pathol. 19, 772 779.

Hardee, M.E., Dewhirst, M.W., Agarwal, N., and Sorg, B.S. (2009). Novel imaging provides new insights into mechanisms of oxygen transport in tumors. Curr. Mol. Med. 9, 435 441.

Harper, J.W., and Elledge, S.J. (2007). The DNA damage response: Ten years after. Mol. Cell 28, 739 745.

Hezel, A.F., and Bardeesy, N. (2008). LKB1; linking cell structure and tumor suppression. Oncogene 27, 6908 6919.

Hlubek, F., Brabletz, T., Budczies, J., Pfeiffer, S., Jung, A., and Kirchner, T. (2007). Heterogeneous expression of Wnt/beta catenin target genes within colorectal cancer. Int. J. Cancer 121, 1941 1948.

Hugo, H., Ackland, M.L., Blick, T., Lawrence, M.G., Clements, J.A., Williams, E.D., and Thompson, E.W. (2007). Epithelial mesenchymal and mesenchymal epithelial transitions in carcinoma progression. J. Cell. Physiol. 213, 374 383.

Hsu, P.P., and Sabatini, D.M. (2008). Cancer cell metabolism: Warburg and beyond. Cell 134, 703 707.

Hynes, N.E., and MacDonald, G. (2009). ErbB receptors and signaling pathways in cancer. Curr. Opin. Cell Biol. 21, 177 184.

Ikushima, H., and Miyazono, K. (2010). TGFbeta signalling: a complex web in cancer progression. Nat. Rev. Cancer 10, 415 424.

Ince, T.A., Richardson, A.L., Bell, G.W., Saitoh, M., Godar, S., Karnoub, A.E., Iglehart, J.D., and Weinberg, R.A. (2007). Transformation of different human breast epithelial cell types leads to distinct tumor phenotypes. Cancer Cell 12, 160 170.

Jackson, S.P., and Bartek, J. (2009). The DNA damage response in human biology and disease. Nature 461, 1071 1078.

Jiang, B.H., and Liu, L.Z. (2009). PI3K/PTEN signaling in angiogenesis and tumorigenesis. Adv. Cancer Res. 102, 19 65.

Johansson, M., Denardo, D.G., and Coussens, L.M. (2008). Polarized immune responses differentially regulate cancer development. Immunol. Rev. 222, 145 154.

Jones, P.A., and Baylin, S.B. (2007). The epigenomics of cancer. Cell 128, 683 692.

Jones, R.G., and Thompson, C.B. (2009). Tumor suppressors and cell metabolism: a recipe for cancer growth. Genes Dev. 23, 537 548.

Joyce, J.A., and Pollard, J.W. (2009). Microenvironmental regulation of metastasis. Nat. Rev. Cancer 9, 239 252.

Junttila, M.R., and Evan, G.I. (2009). p53 a Jack of all trades but master of none. Nat. Rev. Cancer 9, 821 829.

Kalluri, R., and Zeisberg, M. (2006). Fibroblasts in cancer. Nat. Rev. Cancer 6, 392 401.

Kang, H.J., Choi, Y.S., Hong, S.B., Kim, K.W., Woo, R.S., Won, S.J., Kim, E.J., Jeon, H.K., Jo, S.Y., Kim, T.K., et al. (2004). Ectopic expression of the catalytic subunit of telomerase protects against brain injury resulting from ischemia and NMDA induced neurotoxicity. J. Neurosci. 24, 1280 1287.

Karin, M., Lawrence, T., and Nizet, V. (2006). Innate immunity gone awry: linking microbial infections to chronic inflammation and cancer. Cell 124, 823 835.

Karnoub, A.E., and Weinberg, R.A. (2006 2007). Chemokine networks and breast cancer metastasis. Breast Dis. 26, 75 85.

Karnoub, A.E., Dash, A.B., Vo, A.P., Sullivan, A., Brooks, M.W., Bell, G.W., Richardson, A.L., Polyak, K., Tubo, R., and Weinberg, R.A. (2007). Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. Nature 449, 557 563.

Kastan, M.B. (2008). DNA damage responses: mechanisms and roles in human disease: 2007 G.H.A. Clowes Memorial Award Lecture. Mol. Cancer Res. 6, 517 524.

Kawai, T., Hiroi, S., Nakanishi, K., and Meeker, A.K. (2007). Telomere length and telomerase expression in atypical adenomatous hyperplasia and small bronchioloalveolar carcinoma of the lung. Am. J. Clin. Pathol. 127, 254 262.

Kazerounian, S., Yee, K.O., and Lawler, J. (2008). Thrombospondins in cancer. Cell. Mol. Life Sci. 65, 700 712.

Kenific, C.M., Thorburn, A., and Debnath, J. (2010). Autophagy and metastasis: another double edged sword. Curr. Opin. Cell Biol. 22, 241 245.

Kennedy, K.M., and Dewhirst, M.W. (2010). Tumor metabolism of lactate: the influence and therapeutic potential for MCT and CD147 regulation. Future Oncol. 6, 127 148.

Kessenbrock, K., Plaks, V., and Werb, Z. (2010). Matrix metalloproteinases: Regulators of the tumor microenvironment. Cell 141, 52 67.

Kim, M.Y., Oskarsson, T., Acharyya, S., Nguyen, D.X., Zhang, X.H., Norton, L., and Massagué, J. (2009). Tumor self seeding by circulating cancer cells. Cell 139, 1315 1326.

Kim, R., Emi, M., and Tanabe, K. (2007). Cancer immunoediting from immune surveillance to immune escape. Immunology 121, 1 14.

Kinzler, K.W., and Vogelstein, B. (1997). Cancer susceptibility genes. Gatekeepers and caretakers. Nature 386, 761 763.

Klein, C.A. (2009). Parallel progression of primary tumours and metastases. Nat. Rev. Cancer 9, 302 312.

Klymkowsky, M.W., and Savagner, P. (2009). Epithelial mesenchymal transition: a cancer researcher's conceptual friend and foe. Am. J. Pathol. 174, 1588 1593.

Cell

Korkola, J., and Gray, J.W. (2010). Breast cancer genomes form and function. Curr. Opin. Genet. Dev. 20, 4 14.

Kovacic, J.C., and Boehm, M. (2009). Resident vascular progenitor cells: an emerging role for non terminally differentiated vessel resident cells in vascular biology. Stem Cell Res. (Amst.) 2, 2 15.

Kroemer, G., and Pouyssegur, J. (2008). Tumor cell metabolism: Cancer's Achilles' heel. Cancer Cell 13, 472 482.

Lamagna, C., and Bergers, G. (2006). The bone marrow constitutes a reservoir of pericyte progenitors. J. Leukoc. Biol. 80, 677 681.

Lane, D.P. (1992). Cancer. p53, guardian of the genome. Nature 358, 15 16.

Lemmon, M.A., and Schlessinger, J. (2010). Cell signaling by receptor tyrosine kinases. Cell 141, 1117 1134.

Levine, B., and Kroemer, G. (2008). Autophagy in the pathogenesis of disease. Cell 132, 27 42.

Lipinski, M.M., and Jacks, T. (1999). The retinoblastoma gene family in differ entiation and development. Oncogene 18, 7873 7882.

Lobo, N.A., Shimono, Y., Qian, D., and Clarke, M.F. (2007). The biology of cancer stem cells. Annu. Rev. Cell Dev. Biol. 23, 675 699.

Lowe, S.W., Cepero, E., and Evan, G. (2004). Intrinsic tumour suppression. Nature 432, 307 315.

Luebeck, E.G. (2010). Cancer: Genomic evolution of metastasis. Nature 467, 1053 1055.

Lu, Z., Luo, R.Z., Lu, Y., Zhang, X., Yu, Q., Khare, S., Kondo, S., Kondo, Y., Yu, Y., Mills, G.B., et al. (2008). The tumor suppressor gene ARHI regulates au tophagy and tumor dormancy in human ovarian cancer cells. J. Clin. Invest. 118, 3917 3929.

Luo, J., Solimini, N.L., and Elledge, S.J. (2009). Principles of cancer therapy: Oncogene and non oncogene addiction. Cell 136, 823 837.

Mac Gabhann, F., and Popel, A.S. (2008). Systems biology of vascular endo thelial growth factors. Microcirculation 15, 715 738.

Madsen, C.D., and Sahai, E. (2010). Cancer dissemination Lessons from leukocytes. Dev. Cell 19, 13 26.

Maida, Y., Yasukawa, M., Furuuchi, M., Lassmann, T., Possemato, R., Oka moto, N., Kasim, V., Hayashizaki, Y., Hahn, W.C., and Masutomi, K. (2009). An RNA dependent RNA polymerase formed by TERT and the RMRP RNA. Nature 461, 230 235.

Mani, S.A., Guo, W., Liao, M.J., Eaton, E.N., Ayyanan, A., Zhou, A.Y., Brooks, M., Reinhard, F., Zhang, C.C., Shipitsin, M., et al. (2008). The epithelial mesen chymal transition generates cells with properties of stem cells. Cell 133, 704 715.

Mantovani, A. (2010). Molecular pathways linking inflammation and cancer. Curr. Mol. Med. 10, 369 373.

Mantovani, A., Allavena, P., Sica, A., and Balkwill, F. (2008). Cancer related inflammation. Nature 454, 436 444.

Massagué, J. (2008). TGFβ in cancer. Cell 134, 215 230.

Masutomi, K., Possemato, R., Wong, J.M., Currier, J.L., Tothova, Z., Manola, J.B., Ganesan, S., Lansdorp, P.M., Collins, K., and Hahn, W.C. (2005). The telomerase reverse transcriptase regulates chromatin state and DNA damage responses. Proc. Natl. Acad. Sci. USA 102, 8222 8227.

Mathew, R., Karantza Wadsworth, V., and White, E. (2007). Role of autophagy in cancer. Nat. Rev. Cancer 7, 961 967.

McGowan, P.M., Kirstein, J.M., and Chambers, A.F. (2009). Micrometastatic disease and metastatic outgrowth: clinical issues and experimental approaches. Future Oncol. 5, 1083 1098.

Micalizzi, D.S., Farabaugh, S.M., and Ford, H.L. (2010). Epithelial mesen chymal transition in cancer: parallels between normal development and tumor progression. J. Mammary Gland Biol. Neoplasia 15, 117 134.

Mizushima, N. (2007). Autophagy: process and function. Genes Dev. 21, 2861 2873.

Mohamed, M.M., and Sloane, B.F. (2006). Cysteine cathepsins: multifunc tional enzymes in cancer. Nat. Rev. Cancer 6, 764 775.

Mooi, W.J., and Peeper, D.S. (2006). Oncogene induced cell senescence halting on the road to cancer. N. Engl. J. Med. 355, 1037 1046.

Morel, A. P., Lièvre, M., Thomas, C., Hinkal, G., Ansieau, S., and Puisieux, A. (2008). Generation of breast cancer stem cells through epithelial mesen chymal transition. PLoS ONE 3, e2888.

Mosesson, Y., Mills, G.B., and Yarden, Y. (2008). Derailed endocytosis: an emerging feature of cancer. Nat. Rev. Cancer 8, 835 850.

Mougiakakos, D., Choudhury, A., Lladser, A., Kiessling, R., and Johansson, C.C. (2010). Regulatory T cells in cancer. Adv. Cancer Res. 107, 57 117.

Murdoch, C., Muthana, M., Coffelt, S.B., and Lewis, C.E. (2008). The role of myeloid cells in the promotion of tumour angiogenesis. Nat. Rev. Cancer 8, 618 631.

Nagy, J.A., Chang, S.H., Shih, S.C., Dvorak, A.M., and Dvorak, H.F. (2010). Heterogeneity of the tumor vasculature. Semin. Thromb. Hemost. 36, 321 331.

Naumov, G.N., Folkman, J., Straume, O., and Akslen, L.A. (2008). Tumor vascular interactions and tumor dormancy. APMIS 116, 569 585.

Negrini, S., Gorgoulis, V.G., and Halazonetis, T.D. (2010). Genomic insta bility an evolving hallmark of cancer. Nat. Rev. Mol. Cell Biol. 11, 220 228.

Nelson, B.H. (2008). The impact of T cell immunity on ovarian cancer outcomes. Immunol. Rev. 222, 101 116.

Nguyen, D.X., Bos, P.D., and Massagué, J. (2009). Metastasis: from dissemi nation to organ specific colonization. Nat. Rev. Cancer 9, 274 284.

Norden, A.D., Drappatz, J., and Wen, P.Y. (2009). Antiangiogenic therapies for high grade glioma. Nat. Rev. Neurol. 5, 610 620.

Nyberg, P., Xie, L., and Kalluri, R. (2005). Endogenous inhibitors of angiogen esis. Cancer Res. 65, 3967 3979.

Okada, T., Lopez Lago, M., and Giancotti, F.G. (2005). Merlin/NF 2 mediates contact inhibition of growth by suppressing recruitment of Rac to the plasma membrane. J. Cell Biol. 171, 361 371.

Olive, K.P., Jacobetz, M.A., Davidson, C.J., Gopinathan, A., McIntyre, D., Hon ess, D., Madhu, B., Goldgraben, M.A., Caldwell, M.E., Allard, D., et al. (2009). Inhibition of Hedgehog signaling enhances delivery of chemotherapy in a mouse model of pancreatic cancer. Science 324, 1457 1461.

Olson, P., Lu, J., Zhang, H., Shai, A., Chun, M.G., Wang, Y., Libutti, S.K., Na kakura, E.K., Golub, T.R., and Hanahan, D. (2009). MicroRNA dynamics in the stages of tumorigenesis correlate with hallmark capabilities of cancer. Genes Dev. 23, 2152 2165.

O'Reilly, K.E., Rojo, F., She, Q.B., Solit, D., Mills, G.B., Smith, D., Lane, H., Hof mann, F., Hicklin, D.J., Ludwig, D.L., et al. (2006). mTOR inhibition induces upstream receptor tyrosine kinase signaling and activates Akt. Cancer Res. 66, 1500 1508.

Ostrand Rosenberg, S., and Sinha, P. (2009). Myeloid derived suppressor cells: linking inflammation and cancer. J. Immunol. 182, 4499 4506.

Pagès, F., Galon, J., Dieu Nosjean, M.C., Tartour, E., Sautès Fridman, C., and Fridman, W.H. (2010). Immune infiltration in human tumors: a prognostic factor that should not be ignored. Oncogene 29, 1093 1102.

Palermo, C., and Joyce, J.A. (2008). Cysteine cathepsin proteases as pharma cological targets in cancer. Trends Pharmacol. Sci. 29, 22 28.

Park, J.I., Venteicher, A.S., Hong, J.Y., Choi, J., Jun, S., Shkreli, M., Chang, W., Meng, Z., Cheung, P., Ji, H., et al. (2009). Telomerase modulates Wnt signalling by association with target gene chromatin. Nature 460, 66 72.

Partanen, J.I., Nieminen, A.I., and Klefstrom, J. (2009). 3D view to tumor suppression: Lkb1, polarity and the arrest of oncogenic c Myc. Cell Cycle 8, 716 724.

Pasquale, E.B. (2010). Eph receptors and ephrins in cancer: bidirectional sig nalling and beyond. Nat. Rev. Cancer 10, 165 180.

Passos, J.F., Saretzki, G., and von Zglinicki, T. (2007). DNA damage in telo meres and mitochondria during cellular senescence: is there a connection? Nucleic Acids Res. 35, 7505 7513.

Patenaude, A., Parker, J., and Karsan, A. (2010). Involvement of endothelial progenitor cells in tumor vascularization. Microvasc. Res. 79, 217 223.

Cell

Peinado, H., Lavothskin, S., and Lyden, D. (2011). The secreted factors responsible for pre metastatic niche formation: Old sayings and new thoughts. Semin. Cancer Biol. Published online January 18, 2011. 10.1016/j.semcancer.2011.01.002.

Peinado, H., Marin, F., Cubillo, E., Stark, H.J., Fusenig, N., Nieto, M.A., and Cano, A. (2004). Snail and E47 repressors of E cadherin induce distinct inva sive and angiogenic properties in vivo. J. Cell Sci. 117, 2827 2839.

Perona, R. (2006). Cell signalling: growth factors and tyrosine kinase recep tors. Clin. Transl. Oncol. 8, 77 82.

Pietras, K., and Ostman, A. (2010). Hallmarks of cancer: interactions with the tumor stroma. Exp. Cell Res. 316, 1324 1331.

Polyak, K., and Weinberg, R.A. (2009). Transitions between epithelial and mesenchymal states: acquisition of malignant and stem cell traits. Nat. Rev. Cancer 9, 265 273.

Polyak, K., Haviv, I., and Campbell, I.G. (2009). Co evolution of tumor cells and their microenvironment. Trends Genet. 25, 30 38.

Potter, V.R. (1958). The biochemical approach to the cancer problem. Fed. Proc. 17, 691 697.

Qian, B.Z., and Pollard, J.W. (2010). Macrophage diversity enhances tumor progression and metastasis. Cell 141, 39 51.

Quintana, E., Shackleton, M., Sabel, M.S., Fullen, D.R., Johnson, T.M., and Morrison, S.J. (2008). Efficient tumour formation by single human melanoma cells. Nature 456, 593 598.

Raica, M., Cimpean, A.M., and Ribatti, D. (2009). Angiogenesis in pre malig nant conditions. Eur. J. Cancer 45, 1924 1934.

Räsänen, K., and Vaheri, A. (2010). Activation of fibroblasts in cancer stroma. Exp. Cell Res. 316, 2713 2722.

Raynaud, C.M., Hernandez, J., Llorca, F.P., Nuciforo, P., Mathieu, M.C., Commo, F., Delaloge, S., Sabatier, L., André, F., and Soria, J.C. (2010). DNA damage repair and telomere length in normal breast, preoplastic lesions, and invasive cancer. Am. J. Clin. Oncol. 33, 341 345.

Raza, A., Franklin, M.J., and Dudek, A.Z. (2010). Pericytes and vessel matura tion during tumor angiogenesis and metastasis. Am. J. Hematol. 85, 593 598.

Reitman, Z.J., and Yan, H. (2010). Isocitrate dehydrogenase 1 and 2 mutations in cancer: alterations at a crossroads of cellular metabolism. J. Natl. Cancer Inst. 102, 932 941.

Reya, T., Morrison, S.J., Clarke, M.F., and Weissman, I.L. (2001). Stem cells, cancer, and cancer stem cells. Nature 414, 105 111.

Ribatti, D. (2009). Endogenous inhibitors of angiogenesis: a historical review. Leuk. Res. 33, 638 644.

Ricci Vitiani, L., Pallini, R., Biffoni, M., Todaro, M., Invernici, G., Cenci, T., Maira, G., Parati, E.A., Stassi, G., Larocca, L.M., and De Maria, R. (2010). Tumour vascularization via endothelial differentiation of glioblastoma stem like cells. Nature 468, 824 828.

Ruoslahti, E. (2002). Specialization of tumour vasculature. Nat. Rev. Cancer 2, 83 90.

Ruoslahti, E., Bhatia, S.N., and Sailor, M.J. (2010). Targeting of drugs and nanoparticles to tumors. J. Cell Biol. 188, 759 768.

Sabeh, F., Shimizu Hirota, R., and Weiss, S.J. (2009). Protease dependent versus independent cancer cell invasion programs: three dimensional amoe boid movement revisited. J. Cell Biol. 185, 11 19.

Salk, J.J., Fox, E.J., and Loeb, L.A. (2010). Mutational heterogeneity in human cancers: origin and consequences. Ann. Rev. Pathol. 5, 51 75.

Schäfer, M., and Werner, S. (2008). Cancer as an overhealing wound: an old hypothesis revisited. Nat. Rev. Mol. Cell Biol. 9, 628 638.

Schmalhofer, O., Brabletz, S., and Brabletz, T. (2009). E cadherin, beta cate nin, and ZEB1 in malignant progression of cancer. Cancer Metastasis Rev. 28, 151 166.

Semenza, G.L. (2008). Tumor metabolism: cancer cells give and take lactate. J. Clin. Invest. 118, 3835 3837.

Semenza, G.L. (2010a). HIF 1: upstream and downstream of cancer metabo lism. Curr. Opin. Genet. Dev. 20, 51 56.

Semenza, G.L. (2010b). Defining the role of hypoxia inducible factor 1 in cancer biology and therapeutics. Oncogene 29, 625 634.

Seppinen, L., Sormunen, R., Soini, Y., Elamaa, H., Heljasvaara, R., and Pihla janiemi, T. (2008). Lack of collagen XVIII accelerates cutaneous wound healing, while overexpression of its endostatin domain leads to delayed healing. Matrix Biol. 27, 535 546.

Shaw, R.J. (2009). Tumor suppression by LKB1: SIK ness prevents metas tasis. Sci. Signal. 2, pe55.

Shay, J.W., and Wright, W.E. (2000). Hayflick, his limit, and cellular ageing. Nat. Rev. Mol. Cell Biol. 1, 72 76.

Sherr, C.J., and DePinho, R.A. (2000). Cellular senescence: Mitotic clock or culture shock? Cell 102, 407 410.

Sherr, C.J., and McCormick, F. (2002). The RB and p53 pathways in cancer. Cancer Cell 2, 103 112.

Shields, J.D., Kourtis, I.C., Tomei, A.A., Roberts, J.M., and Swartz, M.A. (2010). Induction of lymphoidlike stroma and immune escape by tumors that express the chemokine CCL21. Science 328, 749 752.

Shimoda, M., Mellody, K.T., and Orimo, A. (2010). Carcinoma associated fibroblasts are a rate limiting determinant for tumour progression. Semin. Cell Dev. Biol. 21, 19 25.

Sigal, A., and Rotter, V. (2000). Oncogenic mutations of the p53 tumor suppressor: the demons of the guardian of the genome. Cancer Res. 60, 6788 6793.

Singh, A., and Settleman, J. (2010). EMT, cancer stem cells and drug resis tance: an emerging axis of evil in the war on cancer. Oncogene 29, 4741 4751.

Sinha, S., and Levine, B. (2008). The autophagy effector Beclin 1: a novel BH3 only protein. Oncogene 27 (Suppl 1), S137 S148.

Smyth, M.J., Dunn, G.P., and Schreiber, R.D. (2006). Cancer immunosurveil lance and immunoediting: the roles of immunity in suppressing tumor develop ment and shaping tumor immunogenicity. Adv. Immunol. 90, 1 50.

Soda, Y., Marumoto, T., Friedmann Morvinski, D., Soda, M., Liu, F., Michiue, H., Pastorino, S., Yang, M., Hoffman, R.M., Kesari, S., and Verma, I.M. (2011). Feature Article: Transdifferentiation of glioblastoma cells into vascular endo thelial cells. Proc. Natl. Acad. Sci. USA. Published online January 24, 2011.

Sudarsanam, S., and Johnson, D.E. (2010). Functional consequences of mTOR inhibition. Curr. Opin. Drug Discov. Devel. 13, 31 40.

Strauss, D.C., and Thomas, J.M. (2010). Transmission of donor melanoma by organ transplantation. Lancet Oncol. 11, 790 796.

Talmadge, J.E., and Fidler, I.J. (2010). AACR centennial series: the biology of cancer metastasis: historical perspective. Cancer Res. 70, 5649 5669.

Tammela, T., and Alitalo, K. (2010). Lymphangiogenesis: Molecular mecha nisms and future promise. Cell 140, 460 476.

Taube, J.H., Herschkowitz, J.I., Komurov, K., Zhou, A.Y., Gupta, S., Yang, J., Hartwell, K., Onder, T.T., Gupta, P.B., Evans, K.W., et al. (2010). Core epithe lial to mesenchymal transition interactome gene expression signature is associated with claudin low and metaplastic breast cancer subtypes. Proc. Natl. Acad. Sci. USA 107, 15449 15454.

Teng, M.W.L., Swann, J.B., Koebel, C.M., Schreiber, R.D., and Smyth, M.J. (2008). Immune mediated dormancy: an equilibrium with cancer. J. Leukoc. Biol. 84, 988 993.

Thiery, J.P., and Sleeman, J.P. (2006). Complex networks orchestrate epithe lial mesenchymal transitions. Nat. Rev. Mol. Cell Biol. 7, 131 142.

Thiery, J.P., Acloque, H., Huang, R.Y., and Nieto, M.A. (2009). Epithelial mesenchymal transitions in development and disease. Cell 139, 871 890.

Townson, J.L., and Chambers, A.F. (2006). Dormancy of solitary metastatic cells. Cell Cycle 5, 1744 1750.

Turner, H.E., Harris, A.L., Melmed, S., and Wass, J.A. (2003). Angiogenesis in endocrine tumors. Endocr. Rev. 24, 600 632.

Vajdic, C.M., and van Leeuwen, M.T. (2009). Cancer incidence and risk factors after solid organ transplantation. Int. J. Cancer 125, 1747 1754.

Cell

Vander Heiden, M.G., Cantley, L.C., and Thompson, C.B. (2009). Understanding the Warburg effect: the metabolic requirements of cell proliferation. Science 324, 1029 1033.

Verhoeff, J.J., van Tellingen, O., Claes, A., Stalpers, L.J., van Linde, M.E., Richel, D.J., Leenders, W.P., and van Furth, W.R. (2009). Concerns about anti angiogenic treatment in patients with glioblastoma multiforme. BMC Cancer 9, 444.

Wang, R., Chadalavada, K., Wilshire, J., Kowalik, U., Hovinga, K.E., Geber, A., Fligelman, B., Leversha, M., Brennan, C., and Tabar, V. (2010). Glioblastoma stem like cells give rise to tumour endothelium. Nature 468, 829 833.

Warburg, O. (1956a). On the origin of cancer cells. Science 123, 309 314.

Warburg, O. (1956b). On respiratory impairment in cancer cells. Science 124, 269 270.

Warburg, O.H. (1930). The Metabolism of Tumours: Investigations from the Kaiser Wilhelm Institute for Biology, Berlin Dahlem (London, UK: Arnold Constable).

Wertz, I.E., and Dixit, V.M. (2010). Regulation of death receptor signaling by the ubiquitin system. Cell Death Differ. 17, 14 24.

White, E., Karp, C., Strohecker, A.M., Guo, Y., and Mathew, R. (2010). Role of autophagy in suppression of inflammation and cancer. Curr. Opin. Cell Biol. 22, 212 217.

White, E., and DiPaola, R.S. (2009). The double edged sword of autophagy modulation in cancer. Clin. Cancer Res. 15, 5308 5316.

Willis, S.N., and Adams, J.M. (2005). Life in the balance: how BH3 only proteins induce apoptosis. Curr. Opin. Cell Biol. 17, 617 625.

Witsch, E., Sela, M., and Yarden, Y. (2010). Roles for growth factors in cancer progression. Physiology (Bethesda) 25, 85 101.

Wyckoff, J.B., Wang, Y., Lin, E.Y., Li, J.F., Goswami, S., Stanley, E.R., Segall, J.E., Pollard, J.W., and Condeelis, J. (2007). Direct visualization of macrophage assisted tumor cell intravasation in mammary tumors. Cancer Res. 67, 2649 2656.

Yachida, S., Jones, S., Bozic, I., Antal, T., Leary, R., Fu, B., Kamiyama, M., Hruban, R.H., Eshleman, J.R., Nowak, M.A., et al. (2010). Distant metastasis occurs late during the genetic evolution of pancreatic cancer. Nature 467, 1114 1117.

Yang, J., and Weinberg, R.A. (2008). Epithelial mesenchymal transition: At the crossroads of development and tumor metastasis. Dev. Cell 14, 818 829.

Yang, L., Pang, Y., and Moses, H.L. (2010). TGF beta and immune cells: an important regulatory axis in the tumor microenvironment and progression. Trends Immunol. 31, 220 227.

Yen, K.E., Bittinger, M.A., Su, S.M., and Fantin, V.R. (2010). Cancer associated IDH mutations: biomarker and therapeutic opportunities. Oncogene 29, 6409 6417.

Yilmaz, M., and Christofori, G. (2009). EMT, the cytoskeleton, and cancer cell invasion. Cancer Metastasis Rev. 28, 15 33.

Yuan, T.L., and Cantley, L.C. (2008). PI3K pathway alterations in cancer: variations on a theme. Oncogene 27, 5497 5510.

Zee, Y.K., O'Connor, J.P., Parker, G.J., Jackson, A., Clamp, A.R., Taylor, M.B., Clarke, N.W., and Jayson, G.C. (2010). Imaging angiogenesis of genitourinary tumors. Nat. Rev. Urol. 7, 69 82.

Zhang, H., Herbert, B.S., Pan, K.H., Shay, J.W., and Cohen, S.N. (2004). Disparate effects of telomere attrition on gene expression during replicative senescence of human mammary epithelial cells cultured under different conditions. Oncogene 23, 6193 6198.

Zong, W.X., and Thompson, C.B. (2006). Necrotic death as a cell fate. Genes Dev. 20, 1 15.

Zumsteg, A., and Christofori, G. (2009). Corrupt policemen: inflammatory cells promote tumor angiogenesis. Curr. Opin. Oncol. 21, 60 70.

Exhibit 76

cancers

MDPI

Review

The Role of Inflammation and Inflammatory Mediators in the Development, Progression, Metastasis, and Chemoresistance of Epithelial Ovarian Cancer

Sudha S. Savant [1,†], Shruthi Sriramkumar [2,†] and Heather M. O'Hagan [1,3,*]

[1]   Medical Sciences, Indiana University School of Medicine, Bloomington, IN 47405, USA; ssavant@iu.edu
[2]   Cell, Molecular and Cancer Biology Graduate Program, Indiana University, Bloomington, IN 47405, USA; ssriramk@iu.edu
[3]   Indiana University Melvin and Bren Simon Cancer Center, Indianapolis, IN 46202, USA
[*]   Correspondence: hmohagan@indiana.edu; Tel.: +1-812-855-3035
[†]   These authors contributed equally to this work.

check for updates

Abstract: Inflammation plays a role in the initiation and development of many types of cancers, including epithelial ovarian cancer (EOC) and high grade serous ovarian cancer (HGSC), a type of EOC. There are connections between EOC and both peritoneal and ovulation-induced inflammation. Additionally, EOCs have an inflammatory component that contributes to their progression. At sites of inflammation, epithelial cells are exposed to increased levels of inflammatory mediators such as reactive oxygen species, cytokines, prostaglandins, and growth factors that contribute to increased cell division, and genetic and epigenetic changes. These exposure-induced changes promote excessive cell proliferation, increased survival, malignant transformation, and cancer development. Furthermore, the pro-inflammatory tumor microenvironment environment (TME) contributes to EOC metastasis and chemoresistance. In this review we will discuss the roles inflammation and inflammatory mediators play in the development, progression, metastasis, and chemoresistance of EOC.

Keywords: inflammation; epithelial ovarian cancer; cytokines; reactive oxygen species; growth factors

1. Inflammation and EOC

Inflammation is part of the immune response that protects against foreign pathogens and aids in healing. Inflammation is elicited in response to cellular damage either by infection, exposure to foreign particles (pollutants or irritants), or an increase in cellular stress [1]. The ultimate goal of the inflammatory response is to restore tissue homeostasis, either by destruction or healing of the damaged tissue. The acute or immediate inflammatory response involves modification of the vasculature surrounding the site of stress or damage to increase blood flow. This alteration is then followed by activation of innate immune cells already present in the tissue, including macrophages, dendritic cells (DC), and mast cells, and an increase in infiltration of additional innate immune cells into the affected tissue. At sites of inflammation there are high levels of reactive oxygen species (ROS), cytokines, chemokines, and growth factors that are produced by the immune cells and other cells in the tissue. Acute inflammation is essential for tissue homeostasis and to protect against normal exposure to pathogens. However, in certain cases the body is unable to resolve this response or is subjected to repeated stimulation resulting in chronic inflammation.

Ovarian cancer (OC) is the fifth leading cause of cancer-related deaths in women in the United States [2] and can originate in the germ cells, sex-cord stroma, the fallopian tube (FT), or ovary

epithelium. Epithelial ovarian cancer (EOC) which originates from the ovary or fallopian tube epithelium, accounts for 85–90% of all OCs. Chronic inflammation is an important risk factor associated for EOC and high grade serous ovarian cancer (HGSC), the most malignant subtype of EOC. Chronic inflammation results in activation of signaling pathways, transcription factors, and the innate and adaptive immune responses [3,4]. In this review we primarily focus on inflammation as a risk factor for invasive EOC, but have also included supportive evidence from other OC subtypes, studies that do not define the subtype of OC, and other tumor types as indicated.

1.1. Signaling Pathways and Transcription Factors

Several signaling pathways and transcription factors involved in the inflammatory response also play critical roles in EOC. Here we briefly introduce relevant pathways that will be linked to OC formation in later sections. Cytokines produced during inflammation bind to and activate toll like receptors (TLRs) on cell surfaces, which results in activation of the signaling pathways involving mitogen-activated protein kinases (MAPKs) p38 and JNK (c-Jun N-terminal kinase) and transcription factors including nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and the signal transducer and activator of transcription (STATs). The MAPK pathway regulates cellular processes like proliferation, differentiation, growth, migration, and cell death by upregulating the expression of transcription factors like AP-1, c-Jun, FOS and by activating NF-κB and STATs, that either by themselves or along with AP-1 or c-Jun regulate expression of pro-survival and pro-growth genes. NF-κB and AP-1 also regulate production of cytokines like IL-6 [5–7].

During inflammation these transcription factors play an important role to maintain tissue homeostasis. However, in case of chronic inflammation, the signaling pathways are continuously stimulated, which can contribute to tumorigenesis.

1.2. Innate Immune Response

Inflammation activates the innate immune response, which signals macrophages and DCs to secrete chemoattractants like Interleukin-8 (IL-8), monocyte chemotactic protein-1 (MCP-1), and various other inflammatory mediators. These chemoattractants in turn result in recruitment of neutrophils, lymphocytes, and natural killer (NK) cells to the site of damage. All of these cells then secrete cytokines like IL-1, IL-3, IL-6, IL-8, tumor necrosis factor alpha (TNF-α), interferon (IFN) α, and colony-stimulating factors (CSF) like granulocyte macrophage CSF (GM-CSF). The cytokines bind to transmembrane receptors on the cell surfaces of other cells to activate transcription factors that regulate gene expression downstream of the cytokine activated pathway. This creates a pro-inflammatory environment resulting in recruitment of other immune cells, migration of endothelial cells, and proliferation of fibroblasts. Activation of macrophages and NK cells results in the production of high levels of ROS and reactive nitrogen species (RNS), which are used by these cells to kill foreign pathogens, but also end up damaging neighboring normal cells [8]. The lymphocytes also secrete growth factors like platelet derived growth factor (PDGF), transforming growth factor beta (TGF-β), and fibroblast growth factor (FGF), which facilitate wound healing. Overall the acute immune response is a rapid response that typically only lasts a few days. It results in removal of the pathogen, release of proteolytic enzymes to destroy damaged tissue, or stimulation of the proliferation of fibroblasts and epithelial cells to repair the tissue [1].

1.3. Adaptive Immune Response

If the infection is not resolved by the innate immune response, the adaptive immune response is activated, which is less inflammatory in nature. The adaptive immune response also provides longstanding protection against specific pathogens and/or antigens. B cells and T cells are the effector cells of the adaptive immune system that are derived from lymphocytes when they are presented with specific antigens by the antigen presenting cells (APC). T cells respond to the APCs by producing IL-2, which induces expression of transcription factors that facilitate T cells to differentiate

into T regulatory (Tregs) and T effector (Teff) cells. There are two major classes of T effector cells; CD8+ cytotoxic T cells and CD4+ T helper (Th) cells. Th cells are further differentiated into Th1, Th2, or Th17 depending on the ILs secreted and the transcription factors expressed. IFN-y activates STAT1 to induce formation of Th1 and IL-6, and TGF-β can induce Th17 cell formation. Th1 and Th17 secrete ILs and activate macrophages and B cells to create a pro-inflammatory microenvironment (ME) that can be protumorigenic depending on the context. Tregs are immunosuppressive cells that turn off the immune response [1,9,10].

2. Inflammation as a Risk Factor for EOC

Amongst other factors such as hereditary, environmental, and lifestyle, inflammation emerges as an important risk factor for EOC. EOC arises either in the epithelial layer surrounding the ovary or in the epithelium of the distal FT, which could then spread to the ovary. A significant portion of HGSC is thought to originate in the FT, in part because removal of the FT significantly reduces OC risk [11]. Interestingly, while surgical specimens from mutation carriers rarely had premalignant ovarian epithelial changes, early lesions called serous tubal intraepithelial carcinomas (STICs) were found in the FTs of 5–10% of the patients. Copy number and mutational analysis suggest that STICs shed cells with metastatic potential that then colonize the ovary to form HGSC. STICs are mostly found in the fimbriae, the distal end of the FT that shares a ME with the ovary. During a woman's lifetime, the repeated secretion of ROS, cytokines, and other growth factors by the ovaries and immune cells creates a chronic inflammatory ME in the peritoneum that in turn potentiates the initiation of normal cells to malignant ones in the FT and the ovary, supports tumor progression, metastasis, and development of resistance to chemotherapy.

During ovulation, infection and other causes of inflammation ovary and FT tissue is damaged and undergoes repair. We will briefly discuss how each of these processes evoke or involve an inflammatory response that can persist, leading to a cytokine and growth factor rich environment in the peritoneum and contribute to EOC.

2.1. Ovulation

The process of ovulation itself is comparable to that of inflammation as described in the early 20th century. The development of the follicle to its rupture and release of the egg results in recruitment of activated immune cells to the ovary and production of enormous amounts of chemokines, cytokines, and growth factors. Ovulation is initiated by a surge of Luteinizing hormones (LH) that results in increased blood flow to the ovarian follicles. Before release of the egg, the surge of LH hormone recruits neutrophils and macrophages to the graafian follicles [12–14]. Macrophages in the theca have been shown to support growth of follicles [15]. During ovulation macrophages secrete growth factors like hepatocyte growth factor (HGF), TGF-β, and epidermal growth factor (EGF), which stimulate cellular proliferation and follicle growth. Simultaneously the macrophages also secrete ROS, TNF-α, and IL1β, which stimulate local apoptosis resulting in rupture of the follicle, which bathes the ovarian surface and fimbriae with follicular fluid. Exposure of FT cells to follicular fluid results in altered expression of genes associated with inflammation, including increased expression of IL8 and cyclooxygenase-2 (COX-2) [16]. Quiescent fibroblasts are present in the thecal layer surrounding the follicles. Exposure to growth factors stimulates their proliferation and they then secrete prostaglandins, collagenases, and plasminogen activator. In the corpus luteum, after the follicle is released, the macrophages secrete prostaglandins, ROS, and TNF-α, which stimulate apoptosis of the corpus luteum cells. Therefore, ovulation results in the cyclic exposure of FT and ovarian epithelial cells to high levels of ROS, cytokines, and growth factors [17] Although the other causes of inflammation discussed below are important and result in increased overall risk for EOC, the process of ovulation itself occurs often in the lifetime of the majority of women and may be the most important inflammation-related risk factor for EOC. This hypothesis is corroborated by the laying hen model, which is commonly used to study ovarian cancer [18]. In this model, hens develop spontaneous EOC, likely due to their high ovulation

rate, thus linking ovulation directly as an increased risk factor for EOC. Delayed onset of menarche and early onset of menopause have been shown to be inversely related to the risk of OC, likely due to the reduction in number of ovulation cycles in a woman's lifetime [19,20]. Further, ovulation has also been connected to EOC because contraceptive pills, pregnancy, and breastfeeding reduce the risk of OC. These factors reduce, halt, or delay overall ovulation cycles, respectively, which in turn reduces overall exposure to inflammation of the ovary and FT. The associations of parity and oral contraceptive use with invasive EOC were recently confirmed in a large, prospective study using the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort that found only limited heterogeneity in the risk between reproductive factors and EOC subtypes [21]. Hysterectomy, tube ligation, and removal of ovaries are also protective against development of OC [22,23].

2.2. Infection

Pelvic inflammatory disorder (PID) is the infection of the female reproductive organs like cervix, uterus, FTs, and ovaries. It is a significant risk factor for OC and is caused by various bacteria and virus such as *Chlamydia trachomatis*, *Mycoplasma genitalium*, *Neisseria gonorrhoeae*, human papilloma virus, and cytomegalovirus [24,25]. Infection by these microbes results in DNA damage and production of ROS and induces a pro-inflammatory response, which involves secretion of cytokines and migration of immune cells [24]. PID is generally resolved with antibiotics within 48–72 hours of detection. However, repeated infection and unresolved inflammation can lead to chronic inflammation that is a risk factor for EOC.

2.3. Other Sources of Inflammation

The other causes of inflammation in the ovaries and/or FTs are endometriosis, obesity, Polycystic Ovarian Syndrome (PCOS), and talc exposure. Endometriosis is defined as presence of stroma and endometrial gland tissues in the pelvic peritoneum, rectovaginal septum, and ovaries [26]. Retrograde menstruation is the most commonly accepted theory for endometriosis. Retrograde menstruation results in aberrant accumulation of red blood cells (RBCs) and tissue, which can trigger an inflammatory response, activating the macrophages in the peritoneal cavity [27,28]. The macrophages lyse the RBCs, resulting in an increase in iron accumulation in the endometric implants and peritoneal fluid. The accumulated iron can catalyze formation of free radicals like RNS and ROS in the peritoneum and results in increased oxidative stress (OS). OS can activate NF-κB, in macrophages resulting in secretion of growth factors, cytokines, and IFNs. Around one third of women are affected by mild endometriosis, which resolves on its own over time. For the remaining cases, endometriosis results in chronic pain and inflammation, which can be resolved by excision of affected tissue or the outgrowth. However, in 45% of these cases, the endometriosis reoccurs resulting in repeated bouts of chronic inflammation [29,30].

Obese women have higher risks of EOC and HGSC and pro-inflammatory cytokines are associated with higher body mass index (BMI) levels. Adipose tissues secrete the cytokines TNF-α, IL-6, IL-8, and MCP-1, which can induce an inflammatory reaction in the peritoneum [31]. Continuous secretion of these cytokines leads to a state of chronic inflammation, which includes activation of macrophages and recruitment of NK cells and results in high levels of OS. Once the tumor has been initiated, the continuous secretion of cytokines by adipose tissue or omentum can facilitate migration of cancer cells to the omentum, promoting metastasis of the tumor into the peritoneum [30]. High levels (>10 mg/L) of C-reactive protein (CRP), a marker of global inflammation, are associated with an increased risk of EOC [32,33]. IL-6 itself is not a risk factor for EOC but in obese women IL-6 and CRP may be associated with increased EOC risk [33].

PCOS also contributes to inflammation in women and may increase risk of EOC [34]. PCOS is a hormonal disorder occurring in reproductive aged women during which ovaries may develop numerous small collections of fluid and fail to release eggs properly. Obesity, hyperandrogenism, and increased insulin resistance further characterize PCOS. Increased C-Reactive protein (CRP) and

MCP-1 levels, indicative of low-level chronic inflammation, are elevated in women with PCOS [35–38]. Simultaneously chemokines like IL-18, IL-6, and TNF-α are also increased in circulation in women with PCOS [39–42]. The increase in inflammatory mediators correlates positively with BMI, suggesting that increased obesity in women with PCOS may be the source of inflammation. Increased DNA damage and OS is observed in women with PCOS, which may also increase risk for EOC [43]. Evidence linking PCOS directly to EOC is limited due to small study sizes, PCOS being associated with other EOC risk factors such as obesity, and PCOS possibly being only associated with one subtype of EOC, borderline serous [44].

Talc is a silicate mineral and exposure to it can cause inflammation of the ovaries and poses a risk hazard for development of EOC [45]. It has been proposed that talc from talcum powder used for dusting and from condoms and vaginal diaphragms can migrate up to the ovaries via retrograde flow of fluids and mucous and get lodged in the ovaries. Tubal ligation, which is protective for EOC, is thought to block the transport off talc from the lower genital tract. Talc behaves as a foreign particle, triggering an inflammatory response [46,47]. The talc attracts macrophages, which try to phagocytose it. The macrophages then send chemotactic signals to other immune response mediators and initiate a wound healing process. Since talc is not degradable by the body, it inhibits the wound healing process, resulting in chronic inflammation.

2.4. NSAIDS and Reduced Risk of EOC

Further connecting inflammation to EOC are several studies that demonstrate that intake of non-steroidal anti-inflammatory drugs (NSAIDs), specifically of aspirin, correlates inversely with risk of OC and endometrial cancer [48–52]. In vitro studies with OC cell lines and NSAIDS show that NSAIDs and COX-2 inhibitors facilitate apoptosis, however this effect is not dependent on COX-2 and may be due to upregulation of p21, a protein important for cell cycle arrest [53]. Another study by Arango et al., demonstrates that acetylsalicylic acid or aspirin resulted in increased apoptosis via downregulation of Bcl2 in an endometrial cancer cell line [54]. A third study has shown that a selective COX-2 inhibitor, JTE-522, can inhibit proliferation and increase apoptosis of endometrial cancer cells by increasing levels of p53 and p21 and decreasing phosphorylation of retinoblastoma (Rb) protein, which results in its activation; all of which results in cell cycle arrest [55,56]. Simultaneously, there was an increase in caspase-3 activity, which is indicative of increased apoptosis. Another mechanism by which aspirin could facilitate its chemopreventive nature is by inhibiting oxidative induced DNA damage [57]. COX-1 is also expressed in normal ovaries of the laying hen, with expression increasing in post-ovulatory follicles suggesting its importance for or a role in ovulation. With the onset of OC, COX-1 expression is increased [58] and COX-1 inhibition and NSAIDs have shown to decrease proliferation of ascites in the laying hen OC model [59]. Further, when 0.1% aspirin was included in their diet for one year, although the onset of OC was not different, the progression of cancer was slower when compared to hens fed with regular diet [60].

As discussed, inflammation results in secretion of ROS, growth factors, cytokines, and chemokines into the shared environment surrounding the ovary and distal FT. Exposure of normal tissue to these inflammatory mediators results in activation of downstream signaling that can promote the transformation of normal cells or survival of already transformed cells. Once EOC has already formed further exposure of cancer cells to these inflammatory mediators also results in activation of downstream signaling within the cancer cell and in the surrounding tissue, creating an inflammatory environment that can further promote EOC (Figure 1). We will discuss in more detail how key inflammatory mediators contribute to EOC initiation, progression, metastasis, and chemoresistance.
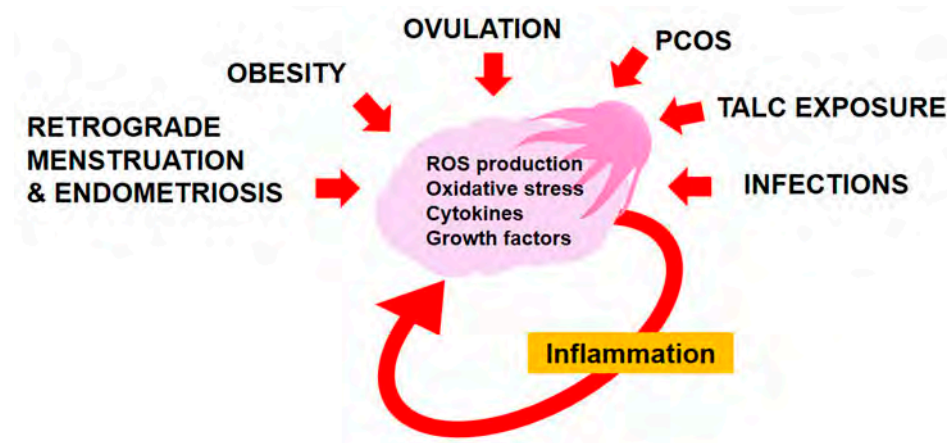
Figure 1. Sources of inflammation in the ovary and fimbriae. Ovulation, retrograde menstruation, endometriosis, infections, exposure to talc, Polycystic Ovarian Syndrome (PCOS), and obesity result in exposure of the ovary and fimbriae to reactive oxygen species (ROS), oxidative stress, cytokines, and growth factors, generating an inflammatory response that leads to additional production of ROS and cytokines in the ovary. Unresolved, chronic inflammation is a critical risk factor for tumor initiation.

3. Inflammation and EOC Initiation and Progression

Tumorigenesis is a multistep process that requires cells to gain the ability to evade apoptosis and antigrowth signals, proliferate independently of stimuli, develop a support system (angiogenesis), and have the capacity to invade and metastasize. Tumorigenesis is initiated by the transformation of a normal cell to a malignant one. The deregulation of the above mentioned processes in the malignant cell could potentiate its progression to cancer.

One mechanism of cancer initiation is genomic instability due to DNA damage [61] and EOCs exhibit a high number of chromosomal aberrations and genomic instability [62]. The most common gene mutations in HGSCs include *BRCA*, *TP53*, and genes in involved in mismatch repair and the DNA damage response [63]. A pro-inflammatory ME can also contribute to genetic instability and therefore play a role in EOC cancer initiation. A pro-inflammatory ME, which is continuously supplemented by ROS, cytokines, and growth factors, can cause DNA damage in epithelial ovarian and FT cells, switch on antiapoptotic pathways, and initiate transformation of normal cells. When cells transformed either by oncogenic alterations or by exposure to inflammation are in a pro-inflammatory ME they are able to turn on pro-survival signaling pathways rather than the senescence pathways that are normally induced by oncogene expression in normal cells. For example, disruption of the RAS pathway results in activated NF-κB signaling and upregulation of its downstream targets including cytokines like IL-1β, IL-6, and IL-8. These cytokines are upregulated in EOC patients and their increased levels correlate with decreased survival [64–71]. The inflammatory mediators like cytokines, chemokines, growth factors, and prostaglandins secreted by the transformed epithelial cells further promote a pro-inflammatory environment, which can reprogram the surrounding cells to form the TME. The TME is mainly composed of endothelial cells, cancer associated fibroblasts (CAFs), adipocytes, tumor associated macrophages (TAMs), regulatory T-cells, pericytes, infiltrated immune cells such as neutrophils, lymphocytes, and various other cells that further secrete growth factors and cytokines which potentiate tumor progression (Figure 2, Table 1). Furthermore, OC-initiating cells (OCICs) have been identified in tumors and ascites that exhibit stem cell like properties and are capable of forming tumors [65,66,72]. Cytokines can promote self-renewal of CD133+ OCICs to potentiate tumor progression [73].
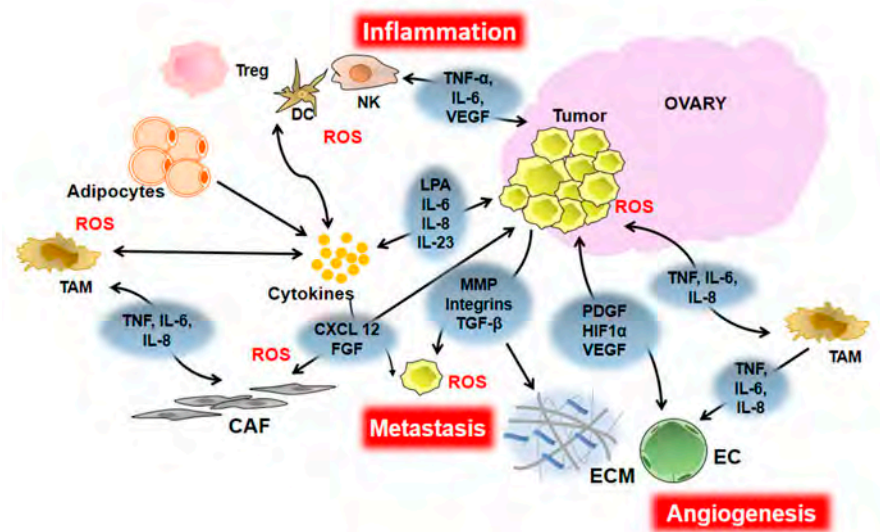
Figure 2. Inflammatory mediators contributing to EOC progression, metastasis, and angiogenesis. EOC cells produce ROS, chemokines, cytokines, and growth factors that can: (1) Lead to recruitment of immune cells like Dentric cells (DC), Natural killer cells (NK), Tumor associated macrophages (TAMs), and T-regulatory (Treg) cells into the TME, which generate additional cytokines, ROS, and growth factors, resulting in chronic inflammation. (2) Stimulate the tumor cells themselves, the TAMs, and the surrounding fibroblasts (also known as cancer associated fibroblasts or CAFs) to proliferate and secrete growth factors like TGF-β and FGF that stimulate production of integrins and Matrix Metalloproteins (MMPs), resulting in migration of the tumor cell via degradation of the extra cellular matrix (ECM). (3) Stimulate endothelial cells (EC) to produce growth factors like PDGF and EGF and factors like VEGF that stimulate angiogenesis. The double arrows indicate that the cells are a source of the factor as well as stimulated by it.

The innate immune response can prevent tumorigenesis by recognizing and eliminating transformed cells. However, chronic inflammation can contribute to the ability of premalignant cells to evade apoptosis, escape the immune surveillance, and continue to grow, resulting in tumor formation. As mentioned, EOC can originate from either distal FT or ovarian epithelial cells. Since both the ovary and fimbria are exposed to the same ME, exposures reviewed here are relevant to initiation in either tissue. [74]. In this section we will review the role of OS and some specific pro-inflammatory mediators and signaling pathways in the initiation and progression of EOC.

3.1. ROS and Oxidative Stress

ROS plays an important role in the normal female reproductive cycle, from affecting maturation of the oocyte to ovulation, apoptosis of cells in corpus luteum, and embryo development [75]. Ovulation results in increased levels of DNA damage in the FT epithelium that is likely a result of the ROS generated during ovulation or the ovulation-associated increase in numbers of infiltrating macrophages in the FT [17]. Additionally, during infection and inflammatory responses immune and damaged cells produce ROS resulting in continuous exposure of the ovaries, FTs, and peritoneal cavity to ROS [76–78]. ROS exposure could potentially lead to epithelial cells in the ovary and FT undergoing transformative changes, as has been demonstrated for ovarian surface epithelium cells grown in 3D culture [79]. Elevated ROS and RNS levels beyond the level that cells can neutralize results in OS. Increased OS results in DNA damage, activation of signaling cascades, and epigenetic alterations.

DNA damage in a cell results in stimulation of DNA damage repair pathways. These repair pathways can be inactivated or be erroneous, which results in increased genotoxic stress and mutated DNA. Secretory tubal epithelial cells in the FT, a cell of origin for HGSC, are particularly susceptible to genotoxic injury with persistent DNA damage that could lead to mutation and STIC formation [80].

Mutations in tumor oncogenes and suppressors result in overexpression, constitutive activation of the protein, loss of expression, or expression of nonfunctional proteins, resulting in a transformed cell. Follicular fluid may have transformative properties as it has been demonstrated that bathing fimbriae with follicular fluid containing high levels of ROS results in increased levels of DNA damage. Bathing fimbriae that have loss of p53 and Rb with this follicular fluid results in evasion of apoptosis and cells with persistent DNA damage [81].

ROS can activate pro-survival intracellular tyrosine phosphorylation signaling cascades, mainly regulated by the MAPKs and redox sensitive kinases. Activation of c-Jun, JNK, ERK (extracellular signal-regulated kinase), and p38-MAPK signaling cascades results in upregulation of cell cycle proteins that enhances proliferation. Activation of JNK can also activate NF-κB, which can suppress apoptosis. The MAPK pathway inhibits apoptosis and regulates differentiation. When activated in transformed cells these pathways are important for tumor initiation. ROS affects redox sensitive factors like thioreoxin, which is also found elevated in OC cell lines [82]. Thioredoxin is involved in redox regulation of transcription factors such as NF-κB, NRF2, forkhead box class O (FOXO) proteins, reducing factor-1 (ref-1), and hypoxia inducible factor (HIF-1α), thereby increasing their binding to the DNA. Most of these transcription factors promote tumor growth and progression by regulating expression of genes that affect cell survival and growth [83,84]. For example, FOXO, NRF2, and ref-1 transcription factors upregulate transcription of anti-oxidant proteins that scavenge free radicals and allow survival of damaged or transformed cells [85]. HIF-1α upregulates the antiapoptotic factor, bcl-2 as well as vascular endothelial growth factor (VEGF), a factor important for angiogenesis.

OS has also been shown to facilitate epigenetic mechanisms in many cancers, including EOC [86]. Innate immune-mediated inflammation drives epigenetic silencing of tumor suppressor genes (TSGs) [87]. At sites of inflammation high levels of OS result in oxidative DNA damage that is recognized by the mismatch repair proteins mutS homolog MSH2 and MSH6. MSH2 and MSH6 then recruit epigenetic silencing proteins, including DNA methyltransferase 1 (DNMT1) to the sites of damage [88]. In an in vivo model of inflammation-driven colon tumorigenesis this early recruitment to sites of oxidative DNA damage results in permanent methylation of TSGs in tumors that form at the sites of inflammation [89]. While such a mechanism has not been directly proven in EOC models, Sapoznik et al. have demonstrated that exposure to follicular fluid or inflammation can induce Activation-Induced Cytidine Deaminase (AIDS) in fallopian tube epithelial cells, which results in epigenetic and genetic changes, increase in DNA damage and genotoxic stress and may be a contributing factor to EOC [90].

3.2. TNF-α

The cytokine TNF-α plays an important role in the process of ovulation and in removal of damaged corpus luteum. TNF-α ligand and its receptors, TNFRI and TNFRII are upregulated in ovarian tumors compared to normal ovarian tissue and high levels of TNF-α are found in ascites from OC patients [91–93]. OC cells have also been shown to secrete high levels of TNF-α as compared to normal ovarian epithelial cells resulting in autocrine upregulation of TNF-α mRNA and in expression of other pro-inflammatory cytokines, chemokines, and angiogenic factors like IL-6, M-CSF, CXCL2, CCL2, and VEGF [93,94]. Kellie et al. have shown using mouse models that TNF-α stimulates IL-17 production via TNFRI resulting in myeloid cell recruitment to the ovarian TME and increased tumor growth [95]. TNF-α, also upregulates AIDS transcript levels which can contribute to genotoxic stress [90].

3.3. IL-6

The cytokine IL-6 has been associated with poor survival in OC and is emerging as a potential therapeutic target for EOC [67,68,96,97]. IL-6 is normally produced by ovarian epithelial and OC cells. Macrophage migration inhibitory factor (MIF), EGF, and Transglutaminase secreted by OC cells can stimulate IL-6 production via activation of NF-κB [98–100]. IL-6 increases proliferation of OC cells by

facilitating their exit from G1 into S phase of the cell cycle and by activation of the MAPK-ERK-Akt (protein kinase B) growth promoting signaling pathway [101]. ERK activation can promote formation of ascites by increasing the migration of tumor cells [70]. IL-6 production by M2 macrophages present in ascites in later stages of EOC can also stimulate cancer cell proliferation via STAT3 activation [102]. High levels of IL-6 can result in immune suppression by downregulation of IL-2, which stimulates Teff cell production [103]. IL-6 also stimulates production of Metallomatrix proteins (MMPs) in OC cells, which increases their invasive properties and promotes tumorigenesis [101,104].

3.4. IL-8

IL-8 a member of C-X-C chemokine family is present in the preovulatory follicle [105] where it may play a role in increasing leukocyte infiltration [106]. It is also elevated in ovarian cysts and in OC patients compared to healthy controls [107,108]. IL-8 has been found to be present in significantly higher levels in the ascites of patients with OC in comparison to patients with benign gynecological disorders [109]. Increased IL-8 expression has been associated with poor prognosis in OC patients [107]. Treatment of EOC cells with IL-8 results in their increased proliferation, which is accompanied by an increase in cyclins B1 and D1 and is dependent on phosphorylation of Akt and ERK [110]. Cyclins B1 and D1 are important for cell cycle progression, and an increase in their expression leads to increased cell growth. On the other hand, two independent studies have demonstrated that IL-8 inhibits EOC growth by increasing neutrophil infiltration [111,112].

3.5. Lyophosphotidic Acid (LPA)

LPA is a phospholipid that binds to and activates the endothelial differentiation gene (Edg) family of receptors. LPA is present in ovarian follicular fluid and it stimulates IL-6 and IL-8 production in the corpus luteum [113,114]. OC cells have been shown to produce LPA, which functions like a growth factor [115–119]. Plasma and ascites of OC patients have elevated levels of LPA that contribute to OC progression via upregulation of COX-2 and MMP2 [115,120,121]. LPA can bind to LPA_2 receptor and induce expression of IL-6 and IL-8 via activation of NF-κB and AP-1 in OC cell lines [122]. It can induce ROS dependent Akt and ERK phosphorylation and inhibition of LPA can increase apoptosis of EOC cells [123]. ERK phosphorylation can induce phosphorylation of HIF-1α, which then can upregulate VEGF and promote tumorigenesis. Another group demonstrated that stimulating EOC cells with ether-linked LPA resulted in their increased proliferation and survival by increased synthesis of DNA and activation of Akt via PI3K, which contributes to tumor progression [124].

3.6. Prostaglandins and COX-1 and COX-2

Prostaglandins are secreted in the ovary, FT, and uterus. They are important for maturation of the oocyte and facilitate the movement of the FT so that the mature oocyte can move from the ovary to the uterus. In the uterus prostaglandins help regulate and maintain uterine blood flow. COX-1 and COX-2 are enzymes that catalyze the production of prostaglandins from arachidonic acid and are overexpressed in OC patients [22,125,126]. High COX levels positively correlate with increased cell proliferation, angiogenesis, and malignancy in ovarian tumors [126,127]. COX-1 and COX-2 are normally involved in the acute inflammatory response but can become dysregulated in chronic inflammatory or TMEs. Obermajer et al. have demonstrated that prostaglandins produced by COX-2 can stimulate production of CXCR4 and its ligand Stromal cell derived factor 1 (SDF1) CXCL12 in myeloid derived suppressor cells (MDSC), which stimulates them to migrate towards OC ascites [128]. MDSCs inhibit the proliferation and differentiation of T cells, resulting in overall immune suppression, which allows the tumor cells to escape immune surveillance and continue to grow. Genetically engineered mouse models of EOC; one harboring the *p53* and *Rb* deletion and other the $KRAS^{G12D}$ mutation and *Pten* deletion, demonstrate increased COX-1 levels, thus suggestive that COX-1 could be used as a potential biomarker and therapeutic target for EOC [129]. Further when

COX-1 was inhibited in EOC cells, it led to reduction in prostacyclin (a type of prostaglandin) synthesis and reduced tumor growth by enhanced apoptosis [130].

4. Inflammation and EOC Angiogenesis

Angiogenesis is required for the growth of both primary and metastatic tumors [131]. The process of angiogenesis is a complex multi-step process reviewed previously [132]. It is regulated by a balance between pro-angiogenic and antiangiogenic factors. Hypoxic and ischemic areas are present at sites of inflammation and also in tumors mainly due to obstruction of local blood vessels, differences in pace of growth of blood vessels and growth of the tumor and/or infiltration of immune cells. Macrophages accumulate at hypoxic sites and alter their gene expression profiles in response to the hypoxic conditions. One of the important genes for angiogenesis that is upregulated by hypoxia is VEGF [133,134]. The rate-limiting step in angiogenesis is VEGF signaling in endothelial cells (ECs) [135]. VEGF functions via tyrosine kinase receptors VEGF-1 and VEGF-2 and promotes migration, survival, proliferation of ECs, and formation of new blood vessels [136–138]. Many of the inflammatory mediators discussed so far are also involved in promoting angiogenesis in EOC as detailed below (Figure 2, Table 1).

4.1. TNF-α

TNF-α creates a pro-inflammatory TME and has also been associated with promoting angiogenesis. It has been hypothesized that TNF-α induces the production of soluble factors that promote tumor angiogenesis. Culture supernatants from TNF-α expressing cells induce the growth of mouse lung endothelial cells in vitro while culture supernatants from TNF-α lacking cells do not exert the same effect [94]. In pituitary adenomas TNF-α is known to induce VEGF that in turn induces CXCL12 [139,140]. VEGF and CXCL12 synergistically induce angiogenesis in EOC [141]. Mice injected with OC cells lacking TNF-α have reduced vascular density in their tumors and reduced formation of blood vessels in the peritoneal deposits. These mice also did not have accumulation of ascetic fluid suggesting the importance of TNF-α in angiogenesis and EOC progression [94].

4.2. IL-6

In physiological conditions, IL-6 is involved in angiogenesis in the ovary during the development of ovarian follicles [142]. IL-6 induces the phosphorylation of STAT3 and MAPK in ovarian endothelial cells thereby enhancing their migratory ability, a key step in angiogenesis [143]. As explained before, OC cells also secrete increased amounts of IL-6. Some OC cells also secrete an alternative splice variant of IL-6Rα, the soluble form sIL-6R, which consists of only the ectodomain of the transmembrane receptor. By a process called trans-signaling, the sIL-6R-IL-6 complex initiates signaling in cells in the ME that do not express the transmembrane receptor facilitating angiogenesis [144].

4.3. IL-8

Several studies have clearly established the role of IL-8 in promoting angiogenesis. Hu et al., demonstrated that IL-8 plays a role in angiogenesis using a rat sponge model [145]. IL-8 was also able to induce angiogenesis in the rat cornea, which is normally avascular [146]. As explained in the previous section, there are several sources of IL-8 in ovarian TME. Overexpression of IL-8 in A2780 (non-IL-8 expressing) OC cells has been shown to increase the expression of VEGF, MMP-2, and MMP-9; while depletion of IL-8 in SKOV3 (IL-8 expressing) cells has been shown to reduce VEGF, MMP-2, and MMP-9 [110]. The process of angiogenesis involves degradation of extracellular matrix components and proliferation and migration of endothelial cells. MMPs are a family of endopeptidases that breakdown components of extracellular matrix and have been implicated in angiogenesis [147]. Because of the importance of VEGF and MMPs in angiogenesis these findings suggest that IL-8 in the ovarian TME will promote the formation of new blood vessels in EOC. Targeting IL-8 using mouse models reduces EOC growth and decreases angiogenesis [112].

Table 1. Role of inflammatory mediators in different stages of tumor progression.

Inflammatory Mediators	Secreting Cell Type	Stages in Tumor Progression			
		Initiation and Progression	Angiogenesis	Metastasis	Chemoresistance
TNF-α ligands, TNFRI, TNFRII	OC cells, infiltrating monocytes, macrophages	↑ autocrine production of TNF-α and IL-6, M-CSF, CXCL2, CCL2 [93,94] and AIDS mRNA level [90]	↑ VEGF, VEGF↑ CXCL12 and promotes angiogenesis [139–141]	↑ TGF-α secretion by stromal fibroblasts which promote peritoneal metastasis [148] Enhances migration of OC cells towards CXCL12 [149,150]	
IL-6	Ovarian epithelial cells, OC cells, M2 macrophages, mesothelial cells, TAMS, ascites	↑Proliferation by promoting G1 to S transition and MAPK-ERK-Akt activation and STAT3 activation [101,102] ↓IL-2, resulting in immune suppression [103]	Induces STAT3 and MAPK phosphorylation which enhances migration of endothelial cells [143] sIL-6R-IL-6 facilitates angiogenesis in cells lacking IL-6 receptor [144]	Stimulates production of MMPs in OCs which ↑ invasion and migration [101,104] ↑ IL-6 in ascites enhances invasion via JAK-STAT signaling [151]	↓ Caspase- 3 cleavage and makes OC cells resistant to cisplatin and paclitaxel [152] ↑Expression of MDR1, GSTpi, Bcl-2, Bcl-xL, and XIAP [152]
IL-8	Pre-ovulatory follicles, OC cells, ascites	↑ Proliferation by ↑ cyclin B1 and cyclin D1 via pAkt [110]	↑ Expression of VEGF, MMP-2, MMP-9 promoting angiogenesis [110]	Activates TAK1/ NF-κB via CXCR2 [153]	Blocks TRAIL induced apoptosis to promote resistance [154]
LPA	Follicular fluid, corpus luteum, OC cells, ascites	↑ IL-6 and IL-8 via NF-κB and AP-1 [113,114,122] ↑COX-2 AND MMP2 [115,120,121] ↑ phosphorylation of Akt and ERK resulting in increased cell cycle [123,124]	↑ Expression of VEGF via Myc and Sp-1 [155]	↑ urokinase, which results in degradation of basemembrane protein to promote metastasis [156,157]	
Prostaglandins, COX-1 and COX-2	Ovary, FT, uterus, MDSCs	↑ CXCR4 and SDF1 in MDSCs resulting in immune suppression [128]	↑ Bcl-2 and blood vessel formation [158,159]		↑ Bcl-2, thus inhibiting apoptosis in lung, colon, breast and prostate cancers [158,159]
TGF-β and EGF	OC cells, CAFs			TGF-β ↑ VCAN, which activates NF-κB and ↑MM-9 [160]	↑ EGF protects cells from cisplatin-induced apoptosis [161]. Inhibiting TGF-β sensitizes resistant cells [162]

4.4. LPA

In addition to playing a role in initiation, and progression, LPA has also been implicated in angiogenesis in OC. LPA has been shown to induce transcriptional activation of VEGF in EOC cell lines [163]. Transcriptional activation of VEGF primarily occurs through HIF-1α under oxygen limiting conditions in Hep3B hepatocellular carcinoma cells [164]. LPA mediated induction of VEGF expression has been shown to be independent of HIF-1α in EOC cell lines. Transition metal cobalt treatment also leads to stabilization of HIF1α similar to hypoxia. Combination treatment of EOC cells with cobalt and LPA additively increased VEGF production suggesting the effect of two different pathways [155]. LPA activates c-Myc and Sp-1, which induce VEGF expression through consensus binding sites in the VEGF promoter that have been implicated in HIFα independent induction of VEGF [155].

5. Inflammation and EOC Metastasis

Tumor metastasis is the major cause of mortality in most cancers, including EOC. Most EOC patients are diagnosed at an advanced stage when the cancer has already metastasized [165]. Dissemination of cancer cells to distant sites is a complex multi-step process called the invasion-metastasis cascade and is reviewed in detail in previous papers [166–168]. Briefly, some major steps in metastasis are—invasion through the basement membrane, intravasation into the lymphatics and circulation, survival of disseminating cancer cells in circulation, extravasation into surrounding tissues, colonization, and finally, formation of micro and macro metastases. However, unlike other epithelial malignancies, EOC has a different pattern of metastasis. EOC cells directly shed from the primary tumor into the peritoneal space and disseminate to organs in the peritoneal cavity. One of the prerequisites for cancer cells to metastasize is to undergo a process called epithelial to mesenchymal transition (EMT) where they lose their ability to attach to the basement membrane and acquire a mesenchymal phenotype and characteristics. Several recent evidences have indicated that the TME aids tumor cells to acquire these properties facilitating the metastatic cascade. An example of the ME promoting metastasis is the presence of STICs in the distal part of the FT, which shares its ME with ovary. Yang-Hartwich et al. have demonstrated that granulosa cells in the ovary secrete SDF-1 (stromal cell-derived factor 1) [169]. SDF-1 functions as a chemoattractant and recruits malignant FT cells to the ovary suggesting that the ovary is a primary site of metastasis, not the primary tumor site. Russo et al. demonstrated that loss of PTEN (phosphatase and tensin homolog) by the malignant FT cells and upregulation of WNT4 (wingless-related MMTV integration site 4) is crucial for initial metastasis to the ovary thereby supporting the tubal origin of EOC and the ovary as the primary site of metastasis [170]. The cells that make up the TME also secrete various inflammatory mediators, which facilitate progression and metastasis of OC cells (Figure 2, Table 1). These factors enable tumor metastasis by deregulating signal transduction pathways. Examples include the PI3-Akt and RAS-ERK pathways, which control migration and invasion through downstream effectors like Rho family GTPases, extracellular proteases, integrins, matrix associated proteins like focal adhesion kinases (FAK), and transcription factors like ETS2 and AP-1 [171–173]. Robinson-Smith et al. demonstrated that peritoneal inflammation correlated with dissemination of cancer cells from the ovaries in SCID mice. Augmenting the inflammatory response using thioglycolate accelerated ascites formation and metastasis while suppressing the inflammation using acetyl salicyclic acid impeded ascites formation and reduced metastasis. This inflammation-induced metastasis of OC cells was found to be primarily mediated by macrophages and not neutrophils or NK cells [174]. As explained in one of the previous sections a pro-inflammatory environment can be created in the peritoneum due to secretion of cytokines like IL-6 and TNF-α by adipose cells [31]. Omentum, the primary site of metastasis of OC, is largely composed of adipose cells. In addition to adipocytes, omentum also consists of blood and lymph vessels, immune cells, and stromal cells [175]. Adipocytes have been shown to increase migration, invasion, and proliferation of EOC cells. Upregulation of SUSD2 a secreted tumor suppressor by adipocytes by guadecitabine treatment reduced EOC migration and invasion. This finding suggests that epigenetic changes in the stromal cells in addition to EOC cells can facilitate EOC

metastasis [176]. Omentum has aggregates of immune cells around the vasculature commonly referred to as milky spots [177]. Melanoma, lung carcinoma, ovarian carcinoma, and mammary carcinoma cell lines have been shown to specifically metastasize to the immune cell aggregates in the omentum when injected intraperitonealy into C57BL/6 mice [178]. These milky spots in the omentum have also been shown to facilitate metastatic colonization of the OC cells. Clark et al. have suggested that both adipocytes and milky spots have specific and important roles in metastatic colonization of OC cells [179]. These evidences imply that omentum potentially provides a good niche for the growth of ovarian cancer cells. Here we will specifically discuss how inflammatory mediators promote tumor metastasis in EOC.

5.1. ROS

EOC cells produce a large amount of ROS [180]. Loss of E-cadherin is one of the characteristic features of tumor cells with increased ability to migrate and invade. Wang et al. demonstrated that ROS leads to HIFα mediated activation of lysl oxidase. Lysl oxidase was shown to inversely correlate with E-cadherin expression promoting migration and invasion in EOC cells [181]. Tumor cells treated with sub-lethal doses of H_2O_2 failed to attach to the extracellular matrix components fibronectin and laminin and had increased metastatic colonization of lung, thereby establishing a role for ROS in tumor cell metastasis [182].

5.2. TNF-α

TNF-α provides a good example of how interactions between cancer and stroma aid in OC metastasis. Ascitic fluid and OCs contain a large number infiltrating macrophages in part because OCs constitutively produce M-CSF, which functions as a chemoattractant for monocytes [183]. These infiltrating monocytes produce many cytokines one of which is TNF-α [184,185]. OC cells also have elevated TNF-α expression that is regulated by DNA hypomethylation and chromatin remodeling of the TNF-α promoter. Increased TNF-α produced by OC cells and macrophages stimulates increased expression of TGF-α in stromal fibroblasts. TGF-α secreting stromal fibroblasts promote peritoneal metastasis of OC via EGF receptor signaling [148].

Furthermore, in EOC cells and clinical biopsies TNF-α expression correlates with one of the most commonly expressed cytokine receptors CXCR4. TNF-α stimulation of EOC cells enhanced their migration toward the only CXCR4 ligand, CXCL12. Stimulation of EOC cells by CXCL12 induced mRNA and protein expression of TNF-α. Therefore, a positive feedback loop has been suggested where in CXCL12 induced TNF-α potentially acts on the cancer cells and induces CXCR4 expression thereby enhancing tumor cell migration [149,150].

5.3. IL-6

IL-6 has also been implicated in metastasis of OC. Elevated levels of IL-6 found in serum and peritoneal fluid of EOC and OC patients have many sources [186–188]. Mesothelial cells in the peritoneum, TAMs, and EOC cells all secrete IL-6 [67]. M2 polarized macrophages in the ovarian TME induce proliferation and invasion of EOC cells by secretion of IL-6 [189]. Increased IL-6 present in ascites from OC patients enhanced the invasive ability of OC cells via the JAK-STAT signaling pathway. Canonically IL-6 signaling occurs by binding of the ligand to its transmembrane receptor IL-6Rα. The effect of IL-6 on invasion of OC cells correlated with their IL-6R expression [151]. Because through trans-signaling, the sIL-6R–IL-6 complex initiates signaling in cells that do not express the transmembrane receptor [144], we hypothesize that IL-6 produced by macrophages could also promote invasion of OC cells similar to the mechanism of induction of angiogenesis.

5.4. IL-8

Increased proliferation, anchorage independent growth, and angiogenic potential are some prerequisites for cells to metastasize. IL-8 increases the proliferation of OC cells and upregulates VEGF

and MMP2 and 9 via activation of NF-κB, which results in enhanced invasive phenotype of OC cells. IL-8 has been shown to activate TAK1/NF-κB signaling via CXCR2, thereby facilitating the seeding and growth of OC cells in the peritoneal cavity during metastasis [153].

5.5. LPA

LPA promotes proliferation, survival, and metastasis of EOC cells by inducing the expression of c-Myc, VEGF, IL-8, MMPs and COX-2 [163,190–193]. LPA acts through its receptors LPAR1-3, which are members of G-protein coupled receptor superfamily. Invasive EOC cells have significantly higher expression of LPAR1 in comparison to non-invasive cell lines and LPA induces EOC cell invasion specifically through LPAR1 and not through LPAR2 or LPAR3 [194]. It can also induce secretion of urokinase in EOC cells, which has been shown to play a role in metastasis and its high levels correlate with advanced OC and poor survival in patients. LPA has been shown to increase promoter activity, mRNA levels, protein levels, and enzyme activity of Urokinase plasminogen activator (uPA) possibly via the edg-4 LPA receptor [156]. uPA is involved in converting plasminogen to plasmin, which facilitates the degradation of basement membrane and extracellular membrane proteins like fibronection aiding in metastasis [157].

5.6. TGF-β

TGF-β initiates signaling by dimerization of serine/threonine kinase receptors. The dimerization of receptors results in their phosphorylation, which then relays signals downstream via SMAD dependent and SMAD independent pathways. Phosphorylation by the TGF-β receptor causes R-SMADs to bind to Co-SMAD and translocate to the nucleus, where they activate transcription of genes that promote invasion, migration. Bone morphogenic proteins (BMPs) are cytokines that belong to TGF-β family and have been associated with progression of many different cancer types. Their mechanism of promoting tumor progression depends on the TME in which the cancer grows and their mode of metastatic spread [195]. Specifically, BMP-2 overexpression has been associated with poor prognosis in OC [196]. Additionally, TGF-β could potentially modify the TME to promote tumorigenesis. Veriscan (VCAN), an extracellular matrix associated protein, was upregulated by TGF-β through TGF-β receptor II (TGFBR2) and SMAD signaling making the EOC cells more aggressive. Increased VCAN expression enhanced motility and invasion of EOC cells by activating NF-κB signaling, increased expression of MMP-9, and hyaluronidase mediated motility receptor [160]. CAFs have higher expression of TGF-β receptors in comparison to normal ovarian fibroblasts and EOC cells suggesting that CAFs within the TME are more responsive to TGF-β then the other cell types [160].

6. Inflammation and EOC Chemoresistance

The standard treatment for EOC patients is cytoreductive surgery followed by platinum/ taxane-based chemotherapy [197]. The main obstacle in treatment of EOC patients is development of chemoresistance. Resistance to chemotherapy can be either intrinsic or acquired. Inherent gene expression patterns harbored by chemo-naïve tumor cells contribute to intrinsic resistance. Acquired resistance is a consequence of different alterations induced after exposure to chemotherapeutic agents [198]. Different mechanisms, including increased drug efflux, decreased uptake of the drug, inactivation of the drug, increased DNA repair, and reduced apoptotic response, have been implicated in development of platinum resistance [199]. Several recent studies have demonstrated that the TME contributes to both intrinsic and acquired resistance. One type of intrinsic drug resistance influenced by the TME is referred to as environment mediated drug resistance (EMDR). In EMDR, factors and cells present in the TME activate diverse signaling events, transiently protecting the tumor cells from undergoing apoptosis in response to chemotherapeutic agents [200,201]. Another type of drug resistance induced by cytokines, chemokines, and growth factors secreted by fibroblast cells in the tumor stroma is called soluble factor mediated drug resistance (SFM-DR). A good example of SFM-DR is IL-6 mediated drug resistance in multiple myeloma. IL-6 is important for growth of multiple

myeloma cells. IL-6 activates STAT3 signaling in these cells and protects them from Fas mediated apoptosis by upregulating antiapoptotic protein Bcl-X_L [202]. Myeloma cells that produced IL-6 in an autocrine manner were found to be resistant to dexamethasone induced apoptosis while non-IL-6 producing cells were sensitive [203]. Cell adhesion mediated drug resistance (CAM-DR) occurs due to adhesion of tumor cells to extracellular matrix components like laminin, collagen, and fibronectin or due to fibroblasts present in the tumor stroma [204]. An example of this type of resistance is when drug sensitive myeloma cells were adhered to an extracellular matrix component fibronectin, they exhibited a reversible drug resistant phenotype which was not due reduced drug accumulation or increase in antiapoptotic proteins like Bcl-X_L [201]. Here we will discuss specific inflammatory mediators and their role in OC chemoresistance (Figure 3).
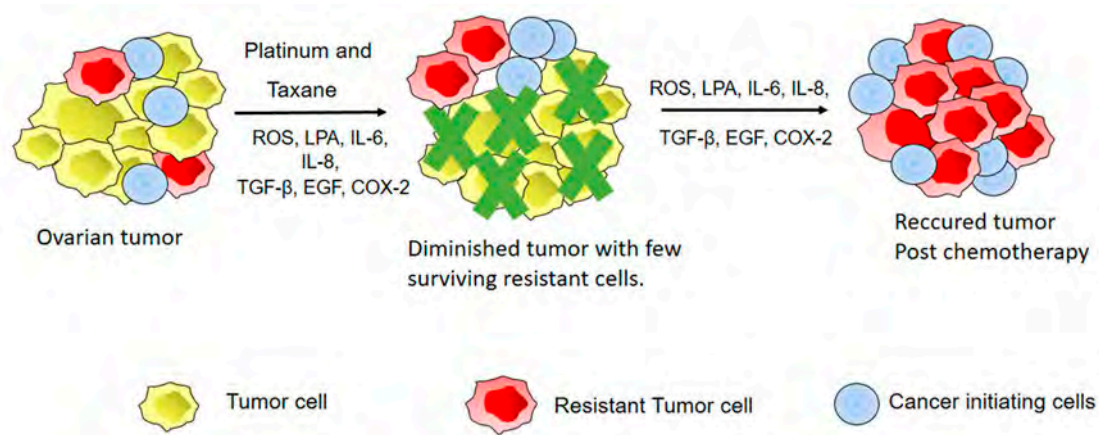
Figure 3. Inflammatory mediators contribute to chemoresistance of EOC. A combination of platinum and taxane drugs is currently used as chemotherapy for OC. ROS, Lyophosphotidic Acid (LPA), cytokines, and growth factors like TGF-β and EGF increase tumor cell survival by upregulating antiapoptotic genes, by stimulating stemness and proliferation of cancer initiating cells, by increasing repair of damaged DNA, or by increasing efflux of the drug. The resistant tumor cells and the cancer initiating cells can then proliferate under the influence of growth factors and cytokines resulting in a recurrent chemoresistant tumor.

6.1. ROS

ROS are abundant in the pro-inflammatory TME. Malignant EOC tissues have been shown to have 96% higher ROS levels than normal controls [205]. OC stem like cells or OCICs are more drug resistant and responsible for relapse of chemoresistant tumors [66]. OCICs produce ROS and superoxide. This ROS induces the expression of peroxisome proliferator-activated receptor-gamma coactivator (PCG)-1α, which regulates mitochondrial biogenesis and is required for expression of detoxifying enzymes [206,207]. PCG1α increases the aldehyde dehydrogenase (ALDH) activity and expression of multidrug resistance gene (MDR1). MDR1 is an ATP dependent transporter that has been associated with efflux of platinum based drugs from OC cells contributing to platinum resistance. Scavenging ROS reduced expression of PCG1α and drug resistant related genes thereby linking ROS to development of chemoresistance [207].

6.2. IL-6

IL-6 in the OC TME is associated with increased chemoresistance. Wang et al. demonstrated that autocrine production of IL-6 by EOC cells makes them resistant to cisplatin and paclitaxel by causing decreased proteolytic cleavage of capase-3. Paclitaxel resistant EOC cells have increased expression of IL-6 and one of its downstream effectors STAT3 [208,209]. IL-6 producing OC cells also had increased expression of multidrug resistant genes MDR1 and GSTpi and anti-apoptotic genes

Bcl-2, Bcl-xL, and XIAP, suggesting that IL6 promotes drug resistance by increasing drug efflux and reducing apoptosis [152].

6.3. IL-8

IL-8 blocks TRAIL-induced apoptosis and reduces caspase cleavage in EOC cell lines by decreasing the expression of death receptor (DR) 4 [210]. TRAIL is a cell death inducing ligand that belongs to the TNF superfamily and has been shown to induce apoptosis specifically in tumor cells and not in nontransformed cells [211,212]. Combination of TRAIL and the chemotherapeutic drugs—cisplatin, doxorubicin, and paclitaxel has been shown to induce apoptosis in chemoresistant EOC cell lines by causing increased caspase and PARP cleavage [154]. This finding suggests that IL8 may contribute to chemoresistance by blocking TRAIL.

6.4. LPA

LPA has been shown to contribute to platinum resistance by preventing cells from undergoing cisplatin-induced apoptosis without affecting their proliferation rate. The mechanism of how LPA inhibits apoptosis in EOC cells in response to cisplatin is not yet clearly understood [161].

6.5. TGF-β and EGF

Recurrent OC show significantly higher expression of TGF-β1 and TGF-β3 in comparison to primary tumors and normal ovary tissue [213]. Inhibition of TGF-β by the inhibitor LY2109761 sensitizes resistant SKOV3 cells to cisplatin suggesting that TGF-β contributes to the development of platinum resistance in EOC cells [162]. Cisplatin resistant A2780P cells had hypomethylation and upregulation of TGFBR2 confirming the involvement of the pathway in acquisition of platinum resistance [214]. An elevated level of EGF receptor (EGFR) has also been associated with poor prognosis in OC patients [215]. EGF has been shown to stimulate the growth of EOC cells expressing EGFR and alters their cell cycle distribution [216]. EGF similar to LPA has been shown to protect EOC cells from undergoing cisplatin induced apoptosis [161].

6.6. COX-2

In addition to being associated with tumor initiation and progression, COX-2 has also been associated with chemoresistance. Ferrandina et al. reported that a statistically significant higher percentage of primary OC patients unresponsive to platinum-containing chemotherapy were positive for COX-2 than responsive patients (84.6% versus 34.6%, respectively) [217]. The percentage of positive COX-2 staining per tumor area in COX-2 positive patients ranged from 15 to 45%. The results from this study suggest that COX-2 levels may influence the response of patients to different chemotherapy regimens, but the sample size of this study was small and the results need to be confirmed in a larger group of patients. Furthermore, this association needs to be corroborated biochemically [217]. In both patients groups undergoing cytoreductive surgery and explorative laparotomy, COX-2 expression was higher in nonresponders [218]. Using lung, colon, and prostate cancer models, COX-2 has been shown to induce Bcl-2 and promote tumor growth by facilitating the formation of new blood vessels [158,159]. These findings suggest that COX-2 may contribute to chemoresistance by inhibiting apoptosis and promoting angiogenesis in OC as well.

7. Treatment Strategies Targeting Inflammatory Mediators in EOC

As discussed, development of resistance to available chemotherapeutic drugs remains the major obstacle in management of OC patients. While several immunotherapies have been developed to improve the antitumor response of T-cells and/or modulate the immune response, here we will discuss EOC treatment strategies that specifically target the inflammatory mediators that have been reviewed above.

A monoclonal antibody directed at VEGF, bevacizumab, has been widely studied and is a promising target in EOC [219]. Bevacizumab is a recombinant humanized monoclonal antibody and has been approved by the FDA for treatment of metastatic breast, non-small cell lung, and colorectal cancer. Phase II clinical studies have shown that it is active in treatment of recurrent OC patients [220]. OCEANS trial was a randomized phase III clinical trial that evaluated the safety and efficacy of bevacizumab in combination with gemcitabine and carboplatin (GC) in comparison with GC alone in recurrent platinum sensitive ovarian, primary peritoneal, or FT cancer. This trial demonstrated that bevacizumab was able to prolong the PFS in platinum-sensitive recurrent EOC patients [221]. In addition to OCEANS, GOG218, and ICON7 have also shown that bevacizumab prolongs the PFS in OC patients confirming the promise this therapeutic target holds for management of OC [222,223].

We have discussed some mechanisms by which the pro-inflammatory cytokine TNF-α promotes OC metastasis and angiogenesis making it a good target for development of therapeutic agents. The safety profile and biological activity of a monoclonal anti-TNF-α antibody, Infliximab was assessed in a clinical study consisting of patients with advanced solid tumors, including OC. Infliximab did not have any toxic effects and was well tolerated by these patients. Reduced plasma levels of IL-6 and CCL12 in these patients was observed 24 h and 48 h after administration of Infliximab, while neutralization of TNF-α was detected after an hour indicating some biological activity [224]. This response warrants further study of Infliximab as a therapeutic agent for treatment of OC.

IL-6/STAT3 signaling has been implicated at different stages of OC progression and is a promising target although most agents are still in preclinical or early clinical trial stages. Siltuximab, an anti-IL-6 antibody, suppresses IL-6-induced STAT3 phosphorylation and nuclear translocation in OC cell lines. Siltuximab treatment also reduced the level of pro-survival proteins like Bcl-X$_L$ and Survivin, which are downstream of STAT3. Siltuximab was able to sensitize paclitaxel resistant OC cell lines, but did not show the same effect in vivo [225]. sc144 is a novel small molecule inhibitor has shown significant promise in preclinical studies. sc144 binds gp130, which is a signal transducer in STAT3 signaling. It causes phosphorylation of gp130 leading to its deglycosylation. This abrogates downstream STAT3 phosphorylation and nuclear translocation inhibiting transcription of downstream genes. sc144 has increased potency in EOC cells in comparison to normal epithelial cells and slows down the growth of tumors in xenograft models of EOC [226]. A phase I clinical trial combining carboplatin, the monoclonal antibody Tocilizumab, which blocks IL-6R, and immune enhancer INF-α showed good promise. The EOC patients who received the highest dose of Tocilizumab had increased serum levels of IL-6 and sIL-6R and also showed longer median overall survival [227].

We have discussed the role of TGF-β in EOC tumor progression substantiating it as a good therapeutic target. A preclinical study of LY2109761 (TGFβRI and TGFβRII kinase inhibitor) in combination with cisplatin was conducted by Gao et al. This inhibitor significantly increased apoptosis in cisplatin resistant cells. Combining LY2109761 with cisplatin had antiproliferative effects and increased the rate of apoptosis in parental and cisplatin resistant xenograft models [162]. In triple negative breast cancer, LY2157299 a TGF-β1 receptor kinase inhibitor, prevented recurrence of tumors in xenograft models after treatment with paclitaxel [228]. Early phase clinical trials of LY2157299 in patients with advanced or metastasized pancreatic cancer have been completed. Early phase trials in triple negative metastatic breast cancer, unresectable hepatocellular carcinoma, and metastatic castration resistant prostate cancer are underway [229].

EGF has also been associated with chemoresistance in EOC. Cetuximab, a chimerized monoclonal antibody that targets EGFR, was tested in combination with carboplatin in patients with recurrent platinum sensitive OC. Cetuximab showed modest activity in these patients [230]. Panitumumab, a human monoclonal antibody specific to EGFR, in combination with carboplatin did not improve efficacy or progression free survival in platinum sensitive EOC patients [231].

8. Conclusions and Future Perspectives

Several studies in the last decade have associated increased inflammation and inflammatory mediators with increased EOC risk and reduced survival in EOC patients. We have presented published evidence suggesting that inflammation and inflammatory mediators promote ovarian tumorigenesis. However the mechanisms by which the process of inflammation culminates in ovarian tumor initiation need to be further understood. Such links have been established in colon and pancreatic cancer. Understanding these mechanisms is important for developing ways to target inflammatory mediators and reduce OC risk. Furthermore, epidemiological studies of NSAIDs and early clinical trials targeting IL-6 and TNF-α have shown significant promise, thus suggesting that targeting inflammatory mediators as treatment for OC warrants future research.

References

1. Maiuri, A.R.; O'Hagan, H.M. Interplay Between Inflammation and Epigenetic Changes in Cancer. *Prog. Mol. Biol. Transl. Sci.* **2016**, *144*, 69–117. [PubMed]
2. Siegel, R.L.; Miller, K.D.; Jemal, A. Cancer statistics, 2018. *CA Cancer J. Clin.* **2018**, *68*, 7–30. [CrossRef] [PubMed]
3. Grivennikov, S.I.; Greten, F.R.; Karin, M. Immunity, inflammation, and cancer. *Cell* **2010**, *140*, 883–899. [CrossRef] [PubMed]
4. Clendenen, T.V.; Lundin, E.; Zeleniuch-Jacquotte, A.; Koenig, K.L.; Berrino, F.; Lukanova, A.; Lokshin, A.E.; Idahl, A.; Ohlson, N.; Hallmans, G.; et al. Circulating inflammation markers and risk of epithelial ovarian cancer. *Cancer Epidemiol. Biomark. Prev.* **2011**, *20*, 799–810. [CrossRef] [PubMed]
5. Chou, C.H.; Wei, L.H.; Kuo, M.L.; Huang, Y.J.; Lai, K.P.; Chen, C.A.; Hsieh, C.Y. Up-regulation of interleukin-6 in human ovarian cancer cell via a Gi/PI3K-Akt/NF-κB pathway by lysophosphatidic acid, an ovarian cancer-activating factor. *Carcinogenesis* **2005**, *26*, 45–52. [CrossRef] [PubMed]
6. Dhillon, A.S.; Hagan, S.; Rath, O.; Kolch, W. MAP kinase signalling pathways in cancer. *Oncogene* **2007**, *26*, 3279–3290. [CrossRef] [PubMed]
7. Schulze-Osthoff, K.; Ferrari, D.; Riehemann, K.; Wesselborg, S. Regulation of NF-κ B activation by MAP kinase cascades. *Immunobiology* **1997**, *198*, 35–49. [CrossRef]
8. Coussens, L.M.; Werb, Z. Inflammation and cancer. *Nature* **2002**, *420*, 860–867. [CrossRef] [PubMed]
9. Gajewski, T.F.; Schreiber, H.; Fu, Y.X. Innate and adaptive immune cells in the tumor microenvironment. *Nat. Immunol.* **2013**, *14*, 1014–1022. [CrossRef] [PubMed]
10. Wilson, C.B.; Rowell, E.; Sekimata, M. Epigenetic control of T-helper-cell differentiation. *Nat. Rev. Immunol.* **2009**, *9*, 91–105. [CrossRef] [PubMed]
11. Tone, A.A.; Salvador, S.; Finlayson, S.J.; Tinker, A.V.; Kwon, J.S.; Lee, C.H.; Cohen, T.; Ehlen, T.; Lee, M.; Carey, M.S.; et al. The role of the fallopian tube in ovarian cancer. *Clin. Adv. Hematol. Oncol.* **2012**, *10*, 296–306. [PubMed]
12. Petrovska, M.; Dimitrov, D.G.; Michael, S.D. Quantitative changes in macrophage distribution in normal mouse ovary over the course of the estrous cycle examined with an image analysis system. *Am. J. Reprod. Immunol.* **1996**, *36*, 175–183. [CrossRef] [PubMed]
13. Takaya, R.; Fukaya, T.; Sasano, H.; Suzuki, T.; Tamura, M.; Yajima, A. Macrophages in normal cycling human ovaries; immunohistochemical localization and characterization. *Hum. Reprod.* **1997**, *12*, 1508–1512. [CrossRef] [PubMed]
14. Wu, R.; Van der Hoek, K.H.; Ryan, N.K.; Norman, R.J.; Robker, R.L. Macrophage contributions to ovarian function. *Hum. Reprod. Update* **2004**, *10*, 119–133. [CrossRef] [PubMed]

15. Tingen, C.M.; Kiesewetter, S.E.; Jozefik, J.; Thomas, C.; Tagler, D.; Shea, L.; Woodruff, T.K. A macrophage and theca cell-enriched stromal cell population influences growth and survival of immature murine follicles in vitro. *Reproduction* **2011**, *141*, 809–820. [CrossRef] [PubMed]

16. Lau, A.; Kollara, A.; St John, E.; Tone, A.A.; Virtanen, C.; Greenblatt, E.M.; King, W.A.; Brown, T.J. Altered expression of inflammation-associated genes in oviductal cells following follicular fluid exposure: Implications for ovarian carcinogenesis. *Exp. Biol. Med.* **2014**, *239*, 24–32. [CrossRef] [PubMed]

17. King, S.M.; Hilliard, T.S.; Wu, L.Y.; Jaffe, R.C.; Fazleabas, A.T.; Burdette, J.E. The impact of ovulation on fallopian tube epithelial cells: Evaluating three hypotheses connecting ovulation and serous ovarian cancer. *Endocr. Relat. Cancer* **2011**, *18*, 627–642. [CrossRef] [PubMed]

18. Johnson, P.A.; Giles, J.R. The hen as a model of ovarian cancer. *Nat. Rev. Cancer* **2013**, *13*, 432–436. [CrossRef] [PubMed]

19. Gong, T.T.; Wu, Q.J.; Vogtmann, E.; Lin, B.; Wang, Y.L. Age at menarche and risk of ovarian cancer: A meta-analysis of epidemiological studies. *Int. J. Cancer* **2013**, *132*, 2894–2900. [CrossRef] [PubMed]

20. Chiaffarino, F.; Pelucchi, C.; Parazzini, F.; Negri, E.; Franceschi, S.; Talamini, R.; Conti, E.; Montella, M.; La Vecchia, C. Reproductive and hormonal factors and ovarian cancer. *Ann. Oncol.* **2001**, *12*, 337–341. [CrossRef] [PubMed]

21. Fortner, R.T.; Ose, J.; Merritt, M.A.; Schock, H.; Tjonneland, A.; Hansen, L.; Overvad, K.; Dossus, L.; Clavel-Chapelon, F.; Baglietto, L.; et al. Reproductive and hormone-related risk factors for epithelial ovarian cancer by histologic pathways, invasiveness and histologic subtypes: Results from the EPIC cohort. *Int. J. Cancer* **2015**, *137*, 1196–1208. [CrossRef] [PubMed]

22. Espey, L.L. Ovulation as an inflammatory reaction—A hypothesis. *Biol. Reprod.* **1980**, *22*, 73–106. [CrossRef] [PubMed]

23. Machelon, V.; Emilie, D. Production of ovarian cytokines and their role in ovulation in the mammalian ovary. *Eur. Cytokine Netw.* **1997**, *8*, 137–143. [PubMed]

24. Chumduri, C.; Gurumurthy, R.K.; Zadora, P.K.; Mi, Y.; Meyer, T.F. Chlamydia infection promotes host DNA damage and proliferation but impairs the DNA damage response. *Cell. Host Microbe* **2013**, *13*, 746–758. [CrossRef] [PubMed]

25. Ingerslev, K.; Hogdall, E.; Schnack, T.H.; Skovrider-Ruminski, W.; Hogdall, C.; Blaakaer, J. The potential role of infectious agents and pelvic inflammatory disease in ovarian carcinogenesis. *Infect. Agent Cancer* **2017**, *12*, 25. [CrossRef] [PubMed]

26. Burney, R.O.; Giudice, L.C. Pathogenesis and pathophysiology of endometriosis. *Fertil. Steril.* **2012**, *98*, 511–519. [CrossRef] [PubMed]

27. Vercellini, P.; Crosignani, P.; Somigliana, E.; Viganò, P.; Buggio, L.; Bolis, G.; Fedele, L. The 'incessant menstruation' hypothesis: A mechanistic ovarian cancer model with implications for prevention. *Hum. Reprod.* **2011**, *26*, 2262–2273. [CrossRef] [PubMed]

28. Burghaus, S.; Haberle, L.; Schrauder, M.G.; Heusinger, K.; Thiel, F.C.; Hein, A.; Wachter, D.; Strehl, J.; Hartmann, A.; Ekici, A.B.; et al. Endometriosis as a risk factor for ovarian or endometrial cancer—Results of a hospital-based case-control study. *BMC Cancer* **2015**, *15*, 751. [CrossRef] [PubMed]

29. Sayasneh, A.; Tsivos, D.; Crawford, R. Endometriosis and ovarian cancer: A systematic review. *ISRN Obstet. Gynecol.* **2011**, *2011*, 140310. [CrossRef] [PubMed]

30. Rizi, B.S.; Nagrath, D. Linking omentum and ovarian cancer: NO. *Oncoscience* **2015**, *2*, 797–798. [PubMed]

31. Gunderson, C.C.; Ding, K.; Dvorak, J.; Moore, K.N.; McMeekin, D.S.; Benbrook, D.M. The pro-inflammatory effect of obesity on high grade serous ovarian cancer. *Gynecol. Oncol.* **2016**, *143*, 40–45. [CrossRef] [PubMed]

32. Poole, E.M.; Lee, I.M.; Ridker, P.M.; Buring, J.E.; Hankinson, S.E.; Tworoger, S.S. A prospective study of circulating C-reactive protein, interleukin-6, and tumor necrosis factor alpha receptor 2 levels and risk of ovarian cancer. *Am. J. Epidemiol.* **2013**, *178*, 1256–1264. [CrossRef] [PubMed]

33. Ose, J.; Schock, H.; Tjonneland, A.; Hansen, L.; Overvad, K.; Dossus, L.; Clavel-Chapelon, F.; Baglietto, L.; Boeing, H.; Trichopoulou, A.; et al. Inflammatory Markers and Risk of Epithelial Ovarian Cancer by Tumor Subtypes: The EPIC Cohort. *Cancer Epidemiol. Biomark. Prev.* **2015**, *24*, 951–961. [CrossRef] [PubMed]

34. Duleba, A.J.; Dokras, A. Is PCOS an inflammatory process? *Fertil. Steril.* **2012**, *97*, 7–12. [CrossRef] [PubMed]

35. Kelly, C.C.; Lyall, H.; Petrie, J.R.; Gould, G.W.; Connell, J.M.; Sattar, N. Low grade chronic inflammation in women with polycystic ovarian syndrome. *J. Clin. Endocrinol. Metab.* **2001**, *86*, 2453–2455. [CrossRef] [PubMed]

36. Escobar-Morreale, H.F.; Luque-Ramirez, M.; Gonzalez, F. Circulating inflammatory markers in polycystic ovary syndrome: A systematic review and metaanalysis. *Fertil. Steril.* **2011**, *95*, 1048–1058.e1041-1042. [CrossRef] [PubMed]

37. Gonzalez, F.; Rote, N.S.; Minium, J.; Kirwan, J.P. Evidence of proatherogenic inflammation in polycystic ovary syndrome. *Metabolism* **2009**, *58*, 954–962. [CrossRef] [PubMed]

38. Glintborg, D.; Andersen, M.; Richelsen, B.; Bruun, J.M. Plasma monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-1α are increased in patients with polycystic ovary syndrome (PCOS) and associated with adiposity, but unaffected by pioglitazone treatment. *Clin. Endocrinol.* **2009**, *71*, 652–658. [CrossRef] [PubMed]

39. Escobar-Morreale, H.F.; Botella-Carretero, J.I.; Villuendas, G.; Sancho, J.; San Millan, J.L. Serum interleukin-18 concentrations are increased in the polycystic ovary syndrome: Relationship to insulin resistance and to obesity. *J. Clin. Endocrinol. Metab.* **2004**, *89*, 806–811. [CrossRef] [PubMed]

40. Yang, Y.; Qiao, J.; Li, R.; Li, M.Z. Is interleukin-18 associated with polycystic ovary syndrome? *Reprod. Biol. Endocrinol.* **2011**, *9*, 7. [CrossRef] [PubMed]

41. Tarkun, I.; Cetinarslan, B.; Turemen, E.; Canturk, Z.; Biyikli, M. Association between Circulating Tumor Necrosis Factor-α, Interleukin-6, and Insulin Resistance in Normal-Weight Women with Polycystic Ovary Syndrome. *Metab. Syndr. Relat. Disord.* **2006**, *4*, 122–128. [CrossRef] [PubMed]

42. Vgontzas, A.N.; Trakada, G.; Bixler, E.O.; Lin, H.M.; Pejovic, S.; Zoumakis, E.; Chrousos, G.P.; Legro, R.S. Plasma interleukin 6 levels are elevated in polycystic ovary syndrome independently of obesity or sleep apnea. *Metabolism* **2006**, *55*, 1076–1082. [CrossRef] [PubMed]

43. Dinger, Y.; Akcay, T.; Erdem, T.; Ilker Saygili, E.; Gundogdu, S. DNA damage, DNA susceptibility to oxidation and glutathione level in women with polycystic ovary syndrome. *Scand. J. Clin Lab. Investig.* **2005**, *65*, 721–728.

44. Harris, H.R.; Terry, K.L. Polycystic ovary syndrome and risk of endometrial, ovarian, and breast cancer: A systematic review. *Fertil. Res. Pract.* **2016**, *2*, 14. [CrossRef] [PubMed]

45. Heller, D.S.; Westhoff, C.; Gordon, R.E.; Katz, N. The relationship between perineal cosmetic talc usage and ovarian talc particle burden. *Am. J. Obstet. Gynecol.* **1996**, *174*, 1507–1510. [CrossRef]

46. Henderson, W.J.; Hamilton, T.C.; Griffiths, K. Talc in normal and malignant ovarian tissue. *Lancet* **1979**, *1*, 499. [CrossRef]

47. Muscat, J.E.; Huncharek, M.S. Perineal talc use and ovarian cancer: A critical review. *Eur J Cancer Prev.* **2008**, *17*, 139–146. [CrossRef] [PubMed]

48. Brasky, T.M.; Moysich, K.B.; Cohn, D.E.; White, E. Non-steroidal anti-inflammatory drugs and endometrial cancer risk in the VITamins And Lifestyle (VITAL) cohort. *Gynecol. Oncol.* **2013**, *128*, 113–119. [CrossRef] [PubMed]

49. Prizment, A.E.; Folsom, A.R.; Anderson, K.E. Nonsteroidal anti-inflammatory drugs and risk for ovarian and endometrial cancers in the Iowa Women's Health Study. *Cancer Epidemiol. Biomark. Prev.* **2010**, *19*, 435–442. [CrossRef] [PubMed]

50. Fairfield, K.M.; Hunter, D.J.; Fuchs, C.S.; Colditz, G.A.; Hankinson, S.E. Aspirin, other NSAIDs, and ovarian cancer risk (United States). *Cancer Causes Control.* **2002**, *13*, 535–542. [CrossRef] [PubMed]

51. Trabert, B.; Poole, E.M.; White, E.; Visvanathan, K.; Adami, H.O.; Anderson, G.L.; Brasky, T.M.; Brinton, L.A.; Fortner, R.T.; Gaudet, M.; et al. Analgesic Use and Ovarian Cancer Risk: An Analysis in the Ovarian Cancer Cohort Consortium. *J. Natl. Cancer Inst.* **2018**. [CrossRef] [PubMed]

52. Peres, L.C.; Camacho, F.; Abbott, S.E.; Alberg, A.J.; Bandera, E.V.; Barnholtz-Sloan, J.; Bondy, M.; Cote, M.L.; Crankshaw, S.; Funkhouser, E.; et al. Analgesic medication use and risk of epithelial ovarian cancer in African American women. *Br. J. Cancer* **2016**, *114*, 819–825. [CrossRef] [PubMed]

53. Rodriguez-Burford, C.; Barnes, M.N.; Oelschlager, D.K.; Myers, R.B.; Talley, L.I.; Partridge, E.E.; Grizzle, W.E. Effects of nonsteroidal anti-inflammatory agents (NSAIDs) on ovarian carcinoma cell lines: Preclinical evaluation of NSAIDs as chemopreventive agents. *Clin. Cancer Res.* **2002**, *8*, 202–209. [PubMed]

54. Arango, H.A.; Icely, S.; Roberts, W.S.; Cavanagh, D.; Becker, J.L. Aspirin effects on endometrial cancer cell growth. *Obstet. Gynecol.* **2001**, *97*, 423–427. [PubMed]

55. Gao, J.; Niwa, K.; Sun, W.; Takemura, M.; Lian, Z.; Onogi, K.; Seishima, M.; Mori, H.; Tamaya, T. Non-steroidal anti-inflammatory drugs inhibit cellular proliferation and upregulate cyclooxygenase-2 protein expression in endometrial cancer cells. *Cancer Sci.* **2004**, *95*, 901–907. [CrossRef] [PubMed]

56. Li, H.L.; Zhang, H.W.; Chen, D.D.; Zhong, L.; Ren, X.D.; St-Tu, R. JTE-522, a selective COX-2 inhibitor, inhibits cell proliferation and induces apoptosis in RL95-2 cells. *Acta Pharmacol Sin.* **2002**, *23*, 631–637. [PubMed]

57. Hsu, C.S.; Li, Y. Aspirin potently inhibits oxidative DNA strand breaks: Implications for cancer chemoprevention. *Biochem. Biophys. Res. Commun.* **2002**, *293*, 705–709. [PubMed]

58. Hales, D.B.; Zhuge, Y.; Lagman, J.A.; Ansenberger, K.; Mahon, C.; Barua, A.; Luborsky, J.L.; Bahr, J.M. Cyclooxygenases expression and distribution in the normal ovary and their role in ovarian cancer in the domestic hen (Gallus domesticus). *Endocrine* **2008**, *33*, 235–244. [CrossRef] [PubMed]

59. Urick, M.E.; Giles, J.R.; Johnson, P.A. VEGF expression and the effect of NSAIDs on ascites cell proliferation in the hen model of ovarian cancer. *Gynecol. Oncol.* **2008**, *110*, 418–424. [CrossRef] [PubMed]

60. Urick, M.E.; Giles, J.R.; Johnson, P.A. Dietary aspirin decreases the stage of ovarian cancer in the hen. *Gynecol. Oncol.* **2009**, *112*, 166–170. [CrossRef] [PubMed]

61. Hanahan, D.; Weinberg, R.A. Hallmarks of cancer: The next generation. *Cell* **2011**, *144*, 646–674. [CrossRef] [PubMed]

62. Cancer Genome Atlas Research, N. Integrated genomic analyses of ovarian carcinoma. *Nature* **2011**, *474*, 609–615.

63. Toss, A.; Tomasello, C.; Razzaboni, E.; Contu, G.; Grandi, G.; Cagnacci, A.; Schilder, R.J.; Cortesi, L. Hereditary ovarian cancer: Not only BRCA 1 and 2 genes. *Biomed. Res. Int.* **2015**, *2015*, 341723. [CrossRef] [PubMed]

64. Liu, J.; Yang, G.; Thompson-Lanza, J.A.; Glassman, A.; Hayes, K.; Patterson, A.; Marquez, R.T.; Auersperg, N.; Yu, Y.; Hahn, W.C.; et al. A genetically defined model for human ovarian cancer. *Cancer Res.* **2004**, *64*, 1655–1663. [CrossRef] [PubMed]

65. Szotek, P.P.; Pieretti-Vanmarcke, R.; Masiakos, P.T.; Dinulescu, D.M.; Connolly, D.; Foster, R.; Dombkowski, D.; Preffer, F.; Maclaughlin, D.T.; Donahoe, P.K. Ovarian cancer side population defines cells with stem cell-like characteristics and Mullerian Inhibiting Substance responsiveness. *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 11154–11159. [CrossRef] [PubMed]

66. Zhang, S.; Balch, C.; Chan, M.W.; Lai, H.C.; Matei, D.; Schilder, J.M.; Yan, P.S.; Huang, T.H.; Nephew, K.P. Identification and characterization of ovarian cancer-initiating cells from primary human tumors. *Cancer Res.* **2008**, *68*, 4311–4320. [CrossRef] [PubMed]

67. Lane, D.; Matte, I.; Rancourt, C.; Piche, A. Prognostic significance of IL-6 and IL-8 ascites levels in ovarian cancer patients. *BMC Cancer* **2011**, *11*, 210. [CrossRef] [PubMed]

68. Masoumi-Moghaddam, S.; Amini, A.; Wei, A.Q.; Robertson, G.; Morris, D.L. Intratumoral interleukin-6 predicts ascites formation in patients with epithelial ovarian cancer: A potential tool for close monitoring. *J. Ovarian Res.* **2015**, *8*, 58. [CrossRef] [PubMed]

69. Rath, K.S.; Funk, H.M.; Bowling, M.C.; Richards, W.E.; Drew, A.F. Expression of soluble interleukin-6 receptor in malignant ovarian tissue. *Am. J. Obstet. Gynecol.* **2010**, *203*, 230.e1–230.e8. [CrossRef] [PubMed]

70. Lo, C.W.; Chen, M.W.; Hsiao, M.; Wang, S.; Chen, C.A.; Hsiao, S.M.; Chang, J.S.; Lai, T.C.; Rose-John, S.; Kuo, M.L.; et al. IL-6 trans-signaling in formation and progression of malignant ascites in ovarian cancer. *Cancer Res.* **2011**, *71*, 424–434. [CrossRef] [PubMed]

71. Dalal, V.; Kumar, R.; Kumar, S.; Sharma, A.; Kumar, L.; Sharma, J.B.; Roy, K.K.; Singh, N.; Vanamail, P. Biomarker potential of IL-6 and VEGF-A in ascitic fluid of epithelial ovarian cancer patients. *Clin. Chim. Acta* **2018**, *482*, 27–32. [CrossRef] [PubMed]

72. Bapat, S.A.; Mali, A.M.; Koppikar, C.B.; Kurrey, N.K. Stem and progenitor-like cells contribute to the aggressive behavior of human epithelial ovarian cancer. *Cancer Res.* **2005**, *65*, 3025–3029. [CrossRef] [PubMed]

73. Wang, D.; Xiang, T.; Zhao, Z.; Lin, K.; Yin, P.; Jiang, L.; Liang, Z.; Zhu, B. Autocrine interleukin-23 promotes self-renewal of CD133+ ovarian cancer stem-like cells. *Oncotarget* **2016**, *7*, 76006–76020. [CrossRef] [PubMed]

74. Maccio, A.; Madeddu, C. Inflammation and ovarian cancer. *Cytokine* **2012**, *58*, 133–147. [CrossRef] [PubMed]

75. Agarwal, A.; Gupta, S.; Sharma, R.K. Role of oxidative stress in female reproduction. *Reprod. Biol. Endocrinol.* **2005**, *3*, 28. [CrossRef] [PubMed]

76. Shkolnik, K.; Tadmor, A.; Ben-Dor, S.; Nevo, N.; Galiani, D.; Dekel, N. Reactive oxygen species are indispensable in ovulation. *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 1462–1467. [CrossRef] [PubMed]

77. Waris, G.; Ahsan, H. Reactive oxygen species: Role in the development of cancer and various chronic conditions. *J. Carcinog.* **2006**, *5*, 14. [CrossRef] [PubMed]

78. Liou, G.Y.; Storz, P. Reactive oxygen species in cancer. *Free Radic. Res.* **2010**, *44*, 479–496. [CrossRef] [PubMed]

79. King, S.M.; Quartuccio, S.M.; Vanderhyden, B.C.; Burdette, J.E. Early transformative changes in normal ovarian surface epithelium induced by oxidative stress require Akt upregulation, DNA damage and epithelial-stromal interaction. *Carcinogenesis* **2013**, *34*, 1125–1133. [CrossRef] [PubMed]

80. Levanon, K.; Ng, V.; Piao, H.Y.; Zhang, Y.; Chang, M.C.; Roh, M.H.; Kindelberger, D.W.; Hirsch, M.S.; Crum, C.P.; Marto, J.A.; et al. Primary ex vivo cultures of human fallopian tube epithelium as a model for serous ovarian carcinogenesis. *Oncogene* **2010**, *29*, 1103–1113. [CrossRef] [PubMed]

81. Huang, H.S.; Chu, S.C.; Hsu, C.F.; Chen, P.C.; Ding, D.C.; Chang, M.Y.; Chu, T.Y. Mutagenic, surviving and tumorigenic effects of follicular fluid in the context of p53 loss: Initiation of fimbria carcinogenesis. *Carcinogenesis* **2015**, *36*, 1419–1428. [CrossRef] [PubMed]

82. Kalinina, E.V.; Chernov, N.N.; Saprin, A.N.; Kotova, Y.N.; Gavrilova, Y.A.; Chermnykh, N.S.; Shcherbak, N.P. Expression of genes for thioredoxin 1 and thioredoxin 2 in multidrug resistance ovarian carcinoma cells SKVLB. *Bull. Exp. Biol. Med.* **2007**, *144*, 301–303. [CrossRef] [PubMed]

83. Ohno, T.; Hirota, K.; Nakamura, H.; Masutani, H.; Sasada, T.; Yodoi, J. Thioredoxin and Its Involvement in the Redox Regulation of Transcription Factors, NF-κB and AP-1. In *Oxygen Homeostasis and Its Dynamics*; Springer: Tokyo, Japan, 1998; pp. 450–456.

84. Van der Wijst, M.G.; Huisman, C.; Mposhi, A.; Roelfes, G.; Rots, M.G. Targeting Nrf2 in healthy and malignant ovarian epithelial cells: Protection versus promotion. *Mol. Oncol.* **2015**, *9*, 1259–1273. [CrossRef] [PubMed]

85. Klotz, L.O.; Sanchez-Ramos, C.; Prieto-Arroyo, I.; Urbanek, P.; Steinbrenner, H.; Monsalve, M. Redox regulation of FoxO transcription factors. *Redox Biol* **2015**, *6*, 51–72. [CrossRef] [PubMed]

86. Ozdemir, F.; Altinisik, J.; Karateke, A.; Coksuer, H.; Buyru, N. Methylation of tumor suppressor genes in ovarian cancer. *Exp. Ther. Med.* **2012**, *4*, 1092–1096. [CrossRef] [PubMed]

87. Niwa, T.; Tsukamoto, T.; Toyoda, T.; Mori, A.; Tanaka, H.; Maekita, T.; Ichinose, M.; Tatematsu, M.; Ushijima, T. Inflammatory processes triggered by Helicobacter pylori infection cause aberrant DNA methylation in gastric epithelial cells. *Cancer Res.* **2010**, *70*, 1430–1440. [CrossRef] [PubMed]

88. Ding, N.; Bonham, E.M.; Hannon, B.E.; Amick, T.R.; Baylin, S.B.; O'Hagan, H.M. Mismatch repair proteins recruit DNA methyltransferase 1 to sites of oxidative DNA damage. *J. Mol. Cell. Biol.* **2016**, *8*, 244–254. [CrossRef] [PubMed]

89. Maiuri, A.R.; Peng, M.; Sriramkumar, S.; Kamplain, C.M.; DeStefano Shields, C.E.; Sears, C.L.; O'Hagan, H.M. Mismatch Repair Proteins Initiate Epigenetic Alterations during Inflammation-Driven Tumorigenesis. *Cancer Res.* **2017**, *77*, 3467–3478. [CrossRef] [PubMed]

90. Sapoznik, S.; Bahar-Shany, K.; Brand, H.; Pinto, Y.; Gabay, O.; Glick-Saar, E.; Dor, C.; Zadok, O.; Barshack, I.; Zundelevich, A.; et al. Activation-Induced Cytidine Deaminase Links Ovulation-Induced Inflammation and Serous Carcinogenesis. *Neoplasia* **2016**, *18*, 90–99. [CrossRef] [PubMed]

91. Gupta, M.; Babic, A.; Beck, A.H.; Terry, K. TNF-α expression, risk factors, and inflammatory exposures in ovarian cancer: Evidence for an inflammatory pathway of ovarian carcinogenesis? *Hum. Pathol.* **2016**, *54*, 82–91. [CrossRef] [PubMed]

92. Moradi, M.M.; Carson, L.F.; Weinberg, B.; Haney, A.F.; Twiggs, L.B.; Ramakrishnan, S. Serum and ascitic fluid levels of interleukin-1, interleukin-6, and tumor necrosis factor-α in patients with ovarian epithelial cancer. *Cancer* **1993**, *72*, 2433–2440. [CrossRef]

93. Szlosarek, P.W.; Grimshaw, M.J.; Kulbe, H.; Wilson, J.L.; Wilbanks, G.D.; Burke, F.; Balkwill, F.R. Expression and regulation of tumor necrosis factor alpha in normal and malignant ovarian epithelium. *Mol. Cancer Ther.* **2006**, *5*, 382–390. [CrossRef] [PubMed]

94. Kulbe, H.; Thompson, R.; Wilson, J.L.; Robinson, S.; Hagemann, T.; Fatah, R.; Gould, D.; Ayhan, A.; Balkwill, F. The inflammatory cytokine tumor necrosis factor-α generates an autocrine tumor-promoting network in epithelial ovarian cancer cells. *Cancer Res.* **2007**, *67*, 585–592. [CrossRef] [PubMed]

95. Charles, K.A.; Kulbe, H.; Soper, R.; Escorcio-Correia, M.; Lawrence, T.; Schultheis, A.; Chakravarty, P.; Thompson, R.G.; Kollias, G.; Smyth, J.F.; et al. The tumor-promoting actions of TNF-α involve TNFR1 and IL-17 in ovarian cancer in mice and humans. *J. Clin. Investig.* **2009**, *119*, 3011–3023. [CrossRef] [PubMed]

96. Berek, J.S.; Chung, C.; Kaldi, K.; Watson, J.M.; Knox, R.M.; Martinez-Maza, O. Serum interleukin-6 levels correlate with disease status in patients with epithelial ovarian cancer. *Am. J. Obstet. Gynecol.* **1991**, *164*, 1038–1042; discussion 1042–1033. [CrossRef]

97. Scambia, G.; Testa, U.; Benedetti Panici, P.; Foti, E.; Martucci, R.; Gadducci, A.; Perillo, A.; Facchini, V.; Peschle, C.; Mancuso, S. Prognostic significance of interleukin 6 serum levels in patients with ovarian cancer. *Br. J. Cancer* **1995**, *71*, 354. [CrossRef] [PubMed]

98. Alberti, C.; Pinciroli, P.; Valeri, B.; Ferri, R.; Ditto, A.; Umezawa, K.; Sensi, M.; Canevari, S.; Tomassetti, A. Ligand-dependent EGFR activation induces the co-expression of IL-6 and PAI-1 via the NFkB pathway in advanced-stage epithelial ovarian cancer. *Oncogene* **2012**, *31*, 4139–4149. [CrossRef] [PubMed]

99. Hagemann, T.; Robinson, S.C.; Thompson, R.G.; Charles, K.; Kulbe, H.; Balkwill, F.R. Ovarian cancer cell-derived migration inhibitory factor enhances tumor growth, progression, and angiogenesis. *Mol. Cancer Ther.* **2007**, *6*, 1993–2002. [CrossRef] [PubMed]

100. Oh, K.; Moon, H.G.; Lee, D.S.; Yoo, Y.B. Tissue transglutaminase-interleukin-6 axis facilitates peritoneal tumor spreading and metastasis of human ovarian cancer cells. *Lab. Anim. Res.* **2015**, *31*, 188–197. [CrossRef] [PubMed]

101. Wang, Y.; Li, L.; Guo, X.; Jin, X.; Sun, W.; Zhang, X.; Xu, R.C. Interleukin-6 signaling regulates anchorage-independent growth, proliferation, adhesion and invasion in human ovarian cancer cells. *Cytokine* **2012**, *59*, 228–236. [CrossRef] [PubMed]

102. Takaishi, K.; Komohara, Y.; Tashiro, H.; Ohtake, H.; Nakagawa, T.; Katabuchi, H.; Takeya, M. Involvement of M2-polarized macrophages in the ascites from advanced epithelial ovarian carcinoma in tumor progression via Stat3 activation. *Cancer Sci.* **2010**, *101*, 2128–2136. [CrossRef] [PubMed]

103. Maccio, A.; Lai, P.; Santona, M.C.; Pagliara, L.; Melis, G.B.; Mantovani, G. High serum levels of soluble IL-2 receptor, cytokines, and C reactive protein correlate with impairment of T cell response in patients with advanced epithelial ovarian cancer. *Gynecol. Oncol.* **1998**, *69*, 248–252. [CrossRef] [PubMed]

104. Rabinovich, A.; Medina, L.; Piura, B.; Segal, S.; Huleihel, M. Regulation of ovarian carcinoma SKOV-3 cell proliferation and secretion of MMPs by autocrine IL-6. *Anticancer Res.* **2007**, *27*, 267–272. [PubMed]

105. Runesson, E.; Bostrom, E.K.; Janson, P.O.; Brannstrom, M. The human preovulatory follicle is a source of the chemotactic cytokine interleukin-8. *Mol. Hum. Reprod* **1996**, *2*, 245–250. [CrossRef] [PubMed]

106. Brannstrom, M.; Mayrhofer, G.; Robertson, S.A. Localization of leukocyte subsets in the rat ovary during the periovulatory period. *Biol. Reprod.* **1993**, *48*, 277–286. [CrossRef] [PubMed]

107. Kassim, S.K.; El-Salahy, E.M.; Fayed, S.T.; Helal, S.A.; Helal, T.; Azzam Eel, D.; Khalifa, A. Vascular endothelial growth factor and interleukin-8 are associated with poor prognosis in epithelial ovarian cancer patients. *Clin. Biochem.* **2004**, *37*, 363–369. [CrossRef] [PubMed]

108. Ivarsson, K.; Runesson, E.; Sundfeldt, K.; Haeger, M.; Hedin, L.; Janson, P.O.; Brannstrom, M. The chemotactic cytokine interleukin-8—A cyst fluid marker for malignant epithelial ovarian cancer? *Gynecol. Oncol.* **1998**, *71*, 420–423. [CrossRef] [PubMed]

109. Radke, J.; Schmidt, D.; Bohme, M.; Schmidt, U.; Weise, W.; Morenz, J. Cytokine level in malignant ascites and peripheral blood of patients with advanced ovarian carcinoma. *Geburtshilfe Frauenheilkd.* **1996**, *56*, 83–87. [CrossRef] [PubMed]

110. Wang, Y.; Xu, R.C.; Zhang, X.L.; Niu, X.L.; Qu, Y.; Li, L.Z.; Meng, X.Y. Interleukin-8 secretion by ovarian cancer cells increases anchorage-independent growth, proliferation, angiogenic potential, adhesion and invasion. *Cytokine* **2012**, *59*, 145–155. [CrossRef] [PubMed]

111. Hirose, K.; Hakozaki, M.; Nyunoya, Y.; Kobayashi, Y.; Matsushita, K.; Takenouchi, T.; Mikata, A.; Mukaida, N.; Matsushima, K. Chemokine gene transfection into tumour cells reduced tumorigenicity in nude mice in association with neutrophilic infiltration. *Br. J. Cancer* **1995**, *72*, 708–714. [CrossRef] [PubMed]

112. Lee, L.F.; Hellendall, R.P.; Wang, Y.; Haskill, J.S.; Mukaida, N.; Matsushima, K.; Ting, J.P. IL-8 reduced tumorigenicity of human ovarian cancer in vivo due to neutrophil infiltration. *J. Immunol.* **2000**, *164*, 2769–2775. [CrossRef] [PubMed]

113. Tokumura, A.; Miyake, M.; Nishioka, Y.; Yamano, S.; Aono, T.; Fukuzawa, K. Production of lysophosphatidic acids by lysophospholipase D in human follicular fluids of In vitro fertilization patients. *Biol. Reprod.* **1999**, *61*, 195–199. [CrossRef] [PubMed]

114. Chen, S.U.; Chou, C.H.; Lee, H.; Ho, C.H.; Lin, C.W.; Yang, Y.S. Lysophosphatidic acid up-regulates expression of interleukin-8 and -6 in granulosa-lutein cells through its receptors and nuclear factor-κB dependent pathways: Implications for angiogenesis of corpus luteum and ovarian hyperstimulation syndrome. *J. Clin. Endocrinol. Metab.* **2008**, *93*, 935–943. [CrossRef] [PubMed]

115. Fang, X.; Gaudette, D.; Furui, T.; Mao, M.; Estrella, V.; Eder, A.; Pustilnik, T.; Sasagawa, T.; Lapushin, R.; Yu, S.; et al. Lysophospholipid growth factors in the initiation, progression, metastases, and management of ovarian cancer. *Ann. N. Y. Acad. Sci.* **2000**, *905*, 188–208. [CrossRef] [PubMed]

116. Xu, Y.; Shen, Z.; Wiper, D.W.; Wu, M.; Morton, R.E.; Elson, P.; Kennedy, A.W.; Belinson, J.; Markman, M.; Casey, G. Lysophosphatidic acid as a potential biomarker for ovarian and other gynecologic cancers. *JAMA* **1998**, *280*, 719–723. [CrossRef] [PubMed]

117. Xiao, Y.J.; Schwartz, B.; Washington, M.; Kennedy, A.; Webster, K.; Belinson, J.; Xu, Y. Electrospray ionization mass spectrometry analysis of lysophospholipids in human ascitic fluids: Comparison of the lysophospholipid contents in malignant vs nonmalignant ascitic fluids. *Anal. Biochem.* **2001**, *290*, 302–313. [CrossRef] [PubMed]

118. Mills, G.B.; Eder, A.; Fang, X.; Hasegawa, Y.; Mao, M.; Lu, Y.; Tanyi, J.; Tabassam, F.H.; Wiener, J.; Lapushin, R.; et al. Critical role of lysophospholipids in the pathophysiology, diagnosis, and management of ovarian cancer. *Cancer Treat. Res.* **2002**, *107*, 259–283. [PubMed]

119. Westermann, A.M.; Havik, E.; Postma, F.R.; Beijnen, J.H.; Dalesio, O.; Moolenaar, W.H.; Rodenhuis, S. Malignant effusions contain lysophosphatidic acid (LPA)-like activity. *Ann. Oncol.* **1998**, *9*, 437–442. [CrossRef] [PubMed]

120. Xu, Y.; Gaudette, D.C.; Boynton, J.D.; Frankel, A.; Fang, X.J.; Sharma, A.; Hurteau, J.; Casey, G.; Goodbody, A.; Mellors, A.; et al. Characterization of an ovarian cancer activating factor in ascites from ovarian cancer patients. *Clin. Cancer Res.* **1995**, *1*, 1223–1232. [PubMed]

121. Symowicz, J.; Adley, B.P.; Woo, M.M.; Auersperg, N.; Hudson, L.G.; Stack, M.S. Cyclooxygenase-2 functions as a downstream mediator of lysophosphatidic acid to promote aggressive behavior in ovarian carcinoma cells. *Cancer Res.* **2005**, *65*, 2234–2242. [CrossRef] [PubMed]

122. Fang, X.; Yu, S.; Bast, R.C.; Liu, S.; Xu, H.J.; Hu, S.X.; LaPushin, R.; Claret, F.X.; Aggarwal, B.B.; Lu, Y.; et al. Mechanisms for lysophosphatidic acid-induced cytokine production in ovarian cancer cells. *J. Biol. Chem.* **2004**, *279*, 9653–9661. [CrossRef] [PubMed]

123. Saunders, J.A.; Rogers, L.C.; Klomsiri, C.; Poole, L.B.; Daniel, L.W. Reactive oxygen species mediate lysophosphatidic acid induced signaling in ovarian cancer cells. *Free Radic. Biol. Med.* **2010**, *49*, 2058–2067. [CrossRef] [PubMed]

124. Lu, J.; Xiao Yj, Y.J.; Baudhuin, L.M.; Hong, G.; Xu, Y. Role of ether-linked lysophosphatidic acids in ovarian cancer cells. *J. Lipid Res.* **2002**, *43*, 463–476. [PubMed]

125. Ali-Fehmi, R.; Morris, R.T.; Bandyopadhyay, S.; Che, M.; Schimp, V.; Malone, J.M., Jr.; Munkarah, A.R. Expression of cyclooxygenase-2 in advanced stage ovarian serous carcinoma: Correlation with tumor cell proliferation, apoptosis, angiogenesis, and survival. *Am. J. Obstet. Gynecol.* **2005**, *192*, 819–825. [CrossRef] [PubMed]

126. Rask, K.; Zhu, Y.; Wang, W.; Hedin, L.; Sundfeldt, K. Ovarian epithelial cancer: A role for PGE2-synthesis and signalling in malignant transformation and progression. *Mol. Cancer* **2006**, *5*, 62. [CrossRef] [PubMed]

127. Heinonen, P.K.; Metsa-Ketela, T. Prostaglandin and thromboxane production in ovarian cancer tissue. *Gynecol. Obstet. Investig.* **1984**, *18*, 225–229. [CrossRef] [PubMed]

128. Obermajer, N.; Muthuswamy, R.; Odunsi, K.; Edwards, R.P.; Kalinski, P. PGE(2)-induced CXCL12 production and CXCR4 expression controls the accumulation of human MDSCs in ovarian cancer environment. *Cancer Res.* **2011**, *71*, 7463–7470. [CrossRef] [PubMed]

129. Daikoku, T.; Tranguch, S.; Trofimova, I.N.; Dinulescu, D.M.; Jacks, T.; Nikitin, A.Y.; Connolly, D.C.; Dey, S.K. Cyclooxygenase-1 is overexpressed in multiple genetically engineered mouse models of epithelial ovarian cancer. *Cancer Res.* **2006**, *66*, 2527–2531. [CrossRef] [PubMed]

130. Li, W.; Cai, J.H.; Zhang, J.; Tang, Y.X.; Wan, L. Effects of cyclooxygenase inhibitors in combination with taxol on expression of cyclin D1 and Ki-67 in a xenograft model of ovarian carcinoma. *Int. J. Mol. Sci.* **2012**, *13*, 9741–9753. [CrossRef] [PubMed]

131. Paweletz, N.; Knierim, M. Tumor-related angiogenesis. *Crit. Rev. Oncol. Hematol.* **1989**, *9*, 197–242. [CrossRef]

132. Potente, M.; Gerhardt, H.; Carmeliet, P. Basic and therapeutic aspects of angiogenesis. *Cell* **2011**, *146*, 873–887. [CrossRef] [PubMed]

133. Burke, B.; Giannoudis, A.; Corke, K.P.; Gill, D.; Wells, M.; Ziegler-Heitbrock, L.; Lewis, C.E. Hypoxia-induced gene expression in human macrophages: Implications for ischemic tissues and hypoxia-regulated gene therapy. *Am. J. Pathol.* **2003**, *163*, 1233–1243. [CrossRef]

134. Murdoch, C.; Muthana, M.; Lewis, C.E. Hypoxia regulates macrophage functions in inflammation. *J. Immunol.* **2005**, *175*, 6257–6263. [CrossRef] [PubMed]

135. Ferrara, N.; Gerber, H.P.; LeCouter, J. The biology of VEGF and its receptors. *Nat. Med.* **2003**, *9*, 669–676. [CrossRef] [PubMed]

136. Wheeler-Jones, C.; Abu-Ghazaleh, R.; Cospedal, R.; Houliston, R.A.; Martin, J.; Zachary, I. Vascular endothelial growth factor stimulates prostacyclin production and activation of cytosolic phospholipase A2 in endothelial cells via p42/p44 mitogen-activated protein kinase. *FEBS Lett.* **1997**, *420*, 28–32. [CrossRef]

137. Gerber, H.P.; McMurtrey, A.; Kowalski, J.; Yan, M.; Keyt, B.A.; Dixit, V.; Ferrara, N. Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3′-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation. *J. Biol. Chem.* **1998**, *273*, 30336–30343. [CrossRef] [PubMed]

138. Gerber, H.P.; Dixit, V.; Ferrara, N. Vascular endothelial growth factor induces expression of the antiapoptotic proteins Bcl-2 and A1 in vascular endothelial cells. *J. Biol. Chem.* **1998**, *273*, 13313–13316. [CrossRef] [PubMed]

139. Xiao, Z.; Liu, Q.; Mao, F.; Wu, J.; Lei, T. TNF-α-induced VEGF and MMP-9 expression promotes hemorrhagic transformation in pituitary adenomas. *Int. J. Mol. Sci.* **2011**, *12*, 4165–4179. [CrossRef] [PubMed]

140. Liang, Z.; Brooks, J.; Willard, M.; Liang, K.; Yoon, Y.; Kang, S.; Shim, H. CXCR4/CXCL12 axis promotes VEGF-mediated tumor angiogenesis through Akt signaling pathway. *Biochem. Biophys. Res. Commun.* **2007**, *359*, 716–722. [CrossRef] [PubMed]

141. Kryczek, I.; Lange, A.; Mottram, P.; Alvarez, X.; Cheng, P.; Hogan, M.; Moons, L.; Wei, S.; Zou, L.; Machelon, V.; et al. CXCL12 and vascular endothelial growth factor synergistically induce neoangiogenesis in human ovarian cancers. *Cancer Res.* **2005**, *65*, 465–472. [PubMed]

142. Motro, B.; Itin, A.; Sachs, L.; Keshet, E. Pattern of interleukin 6 gene expression in vivo suggests a role for this cytokine in angiogenesis. *Proc. Natl. Acad. Sci. USA* **1990**, *87*, 3092–3096. [CrossRef] [PubMed]

143. Nilsson, M.B.; Langley, R.R.; Fidler, I.J. Interleukin-6, secreted by human ovarian carcinoma cells, is a potent proangiogenic cytokine. *Cancer Res.* **2005**, *65*, 10794–10800. [CrossRef] [PubMed]

144. Rose-John, S.; Scheller, J.; Elson, G.; Jones, S.A. Interleukin-6 biology is coordinated by membrane-bound and soluble receptors: Role in inflammation and cancer. *J. Leukocyte Biol.* **2006**, *80*, 227–236. [CrossRef] [PubMed]

145. Hu, D.E.; Hori, Y.; Fan, T.P. Interleukin-8 stimulates angiogenesis in rats. *Inflammation* **1993**, *17*, 135–143. [CrossRef] [PubMed]

146. Koch, A.E.; Polverini, P.J.; Kunkel, S.L.; Harlow, L.A.; DiPietro, L.A.; Elner, V.M.; Elner, S.G.; Strieter, R.M. Interleukin-8 as a macrophage-derived mediator of angiogenesis. *Science* **1992**, *258*, 1798–1801. [CrossRef] [PubMed]

147. Stetler-Stevenson, W.G. Matrix metalloproteinases in angiogenesis: A moving target for therapeutic intervention. *J. Clin. Investig.* **1999**, *103*, 1237–1241. [CrossRef] [PubMed]

148. Lau, T.S.; Chan, L.K.; Wong, E.C.; Hui, C.W.; Sneddon, K.; Cheung, T.H.; Yim, S.F.; Lee, J.H.; Yeung, C.S.; Chung, T.K.; et al. A loop of cancer-stroma-cancer interaction promotes peritoneal metastasis of ovarian cancer via TNFα-TGFα-EGFR. *Oncogene* **2017**, *36*, 3576–3587. [CrossRef] [PubMed]

149. Scotton, C.J.; Wilson, J.L.; Scott, K.; Stamp, G.; Wilbanks, G.D.; Fricker, S.; Bridger, G.; Balkwill, F.R. Multiple actions of the chemokine CXCL12 on epithelial tumor cells in human ovarian cancer. *Cancer Res.* **2002**, *62*, 5930–5938. [PubMed]

150. Kulbe, H.; Hagemann, T.; Szlosarek, P.W.; Balkwill, F.R.; Wilson, J.L. The inflammatory cytokine tumor necrosis factor-α regulates chemokine receptor expression on ovarian cancer cells. *Cancer Res.* **2005**, *65*, 10355–10362. [CrossRef] [PubMed]

151. Kim, S.; Gwak, H.; Kim, H.S.; Kim, B.; Dhanasekaran, D.N.; Song, Y.S. Malignant ascites enhances migratory and invasive properties of ovarian cancer cells with membrane bound IL-6R in vitro. *Oncotarget* **2016**, *7*, 83148–83159. [CrossRef] [PubMed]

152. Wang, Y.; Niu, X.L.; Qu, Y.; Wu, J.; Zhu, Y.Q.; Sun, W.J.; Li, L.Z. Autocrine production of interleukin-6 confers cisplatin and paclitaxel resistance in ovarian cancer cells. *Cancer Lett.* **2010**, *295*, 110–123. [CrossRef] [PubMed]

153. Yung, M.M.; Tang, H.W.; Cai, P.C.; Leung, T.H.; Ngu, S.F.; Chan, K.K.; Xu, D.; Yang, H.; Ngan, H.Y.; Chan, D.W. GRO-α and IL-8 enhance ovarian cancer metastatic potential via the CXCR2-mediated TAK1/NFκB signaling cascade. *Theranostics* **2018**, *8*, 1270–1285. [CrossRef] [PubMed]

154. Cuello, M.; Ettenberg, S.A.; Nau, M.M.; Lipkowitz, S. Synergistic induction of apoptosis by the combination of trail and chemotherapy in chemoresistant ovarian cancer cells. *Gynecol. Oncol.* **2001**, *81*, 380–390. [CrossRef] [PubMed]

155. Song, Y.; Wu, J.; Oyesanya, R.A.; Lee, Z.; Mukherjee, A.; Fang, X. Sp-1 and c-Myc mediate lysophosphatidic acid-induced expression of vascular endothelial growth factor in ovarian cancer cells via a hypoxia-inducible factor-1-independent mechanism. *Clin. Cancer Res.* **2009**, *15*, 492–501. [CrossRef] [PubMed]

156. Pustilnik, T.B.; Estrella, V.; Wiener, J.R.; Mao, M.; Eder, A.; Watt, M.A.; Bast, R.C., Jr.; Mills, G.B. Lysophosphatidic acid induces urokinase secretion by ovarian cancer cells. *Clin. Cancer Res.* **1999**, *5*, 3704–3710. [PubMed]

157. Duffy, M.J. Proteases as prognostic markers in cancer. *Clin. Cancer Res.* **1996**, *2*, 613–618. [PubMed]

158. Masferrer, J.L.; Leahy, K.M.; Koki, A.T.; Zweifel, B.S.; Settle, S.L.; Woerner, B.M.; Edwards, D.A.; Flickinger, A.G.; Moore, R.J.; Seibert, K. Antiangiogenic and antitumor activities of cyclooxygenase-2 inhibitors. *Cancer Res.* **2000**, *60*, 1306–1311. [PubMed]

159. Liu, X.H.; Yao, S.; Kirschenbaum, A.; Levine, A.C. NS398, a selective cyclooxygenase-2 inhibitor, induces apoptosis and down-regulates bcl-2 expression in LNCaP cells. *Cancer Res.* **1998**, *58*, 4245–4249. [PubMed]

160. Yeung, T.L.; Leung, C.S.; Wong, K.K.; Samimi, G.; Thompson, M.S.; Liu, J.; Zaid, T.M.; Ghosh, S.; Birrer, M.J.; Mok, S.C. TGF-beta modulates ovarian cancer invasion by upregulating CAF-derived versican in the tumor microenvironment. *Cancer Res.* **2013**, *73*, 5016–5028. [CrossRef] [PubMed]

161. Frankel, A.; Mills, G.B. Peptide and lipid growth factors decrease cis-diamminedichloroplatinum-induced cell death in human ovarian cancer cells. *Clin. Cancer Res.* **1996**, *2*, 1307–1313. [PubMed]

162. Gao, Y.; Shan, N.; Zhao, C.; Wang, Y.; Xu, F.; Li, J.; Yu, X.; Gao, L.; Yi, Z. LY2109761 enhances cisplatin antitumor activity in ovarian cancer cells. *Int J. Clin. Exp. Pathol.* **2015**, *8*, 4923–4932. [PubMed]

163. Hu, Y.L.; Tee, M.K.; Goetzl, E.J.; Auersperg, N.; Mills, G.B.; Ferrara, N.; Jaffe, R.B. Lysophosphatidic acid induction of vascular endothelial growth factor expression in human ovarian cancer cells. *J. Natl. Cancer Inst.* **2001**, *93*, 762–768. [CrossRef] [PubMed]

164. Forsythe, J.A.; Jiang, B.H.; Iyer, N.V.; Agani, F.; Leung, S.W.; Koos, R.D.; Semenza, G.L. Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. *Mol. Cell. Biol.* **1996**, *16*, 4604–4613. [CrossRef] [PubMed]

165. Tan, D.S.; Agarwal, R.; Kaye, S.B. Mechanisms of transcoelomic metastasis in ovarian cancer. *Lancet Oncol.* **2006**, *7*, 925–934. [CrossRef]

166. Fidler, I.J. The pathogenesis of cancer metastasis: The 'seed and soil' hypothesis revisited. *Nat. Rev. Cancer* **2003**, *3*, 453–458. [CrossRef] [PubMed]

167. Gupta, G.P.; Massague, J. Cancer metastasis: Building a framework. *Cell* **2006**, *127*, 679–695. [CrossRef] [PubMed]

168. Talmadge, J.E.; Fidler, I.J. AACR centennial series: The biology of cancer metastasis: Historical perspective. *Cancer Res.* **2010**, *70*, 5649–5669. [CrossRef] [PubMed]

169. Yang-Hartwich, Y.; Gurrea-Soteras, M.; Sumi, N.; Joo, W.D.; Holmberg, J.C.; Craveiro, V.; Alvero, A.B.; Mor, G. Ovulation and extra-ovarian origin of ovarian cancer. *Sci. Rep.* **2014**, *4*, 6116. [CrossRef] [PubMed]

170. Russo, A.; Czarnecki, A.A.; Dean, M.; Modi, D.A.; Lantvit, D.D.; Hardy, L.; Baligod, S.; Davis, D.A.; Wei, J.J.; Burdette, J.E. PTEN loss in the fallopian tube induces hyperplasia and ovarian tumor formation. *Oncogene* **2018**, *37*, 1976–1990. [CrossRef] [PubMed]

171. Cain, R.J.; Ridley, A.J. Phosphoinositide 3-kinases in cell migration. *Biol. Cell* **2009**, *101*, 13–29. [CrossRef] [PubMed]

172. Raftopoulou, M.; Hall, A. Cell migration: Rho GTPases lead the way. *Dev. Biol.* **2004**, *265*, 23–32. [CrossRef] [PubMed]

173. Devreotes, P.; Horwitz, A.R. Signaling networks that regulate cell migration. *Cold Spring Harb Perspect. Biol.* **2015**, *7*, a005959. [CrossRef] [PubMed]

174. Robinson-Smith, T.M.; Isaacsohn, I.; Mercer, C.A.; Zhou, M.; Van Rooijen, N.; Husseinzadeh, N.; McFarland-Mancini, M.M.; Drew, A.F. Macrophages mediate inflammation-enhanced metastasis of ovarian tumors in mice. *Cancer Res.* **2007**, *67*, 5708–5716. [CrossRef] [PubMed]

175. Wilkosz, S.; Ireland, G.; Khwaja, N.; Walker, M.; Butt, R.; de Giorgio-Miller, A.; Herrick, S.E. A comparative study of the structure of human and murine greater omentum. *Anat. Embryol.* **2005**, *209*, 251–261. [CrossRef] [PubMed]

176. Tang, J.; Pulliam, N.; Ozes, A.; Buechlein, A.; Ding, N.; Keer, H.; Rusch, D.; O'Hagan, H.; Stack, M.S.; Nephew, K.P. Epigenetic Targeting of Adipocytes Inhibits High-Grade Serous Ovarian Cancer Cell Migration and Invasion. *Mol. Cancer Res.* **2018**. [CrossRef] [PubMed]

177. Meza-Perez, S.; Randall, T.D. Immunological Functions of the Omentum. *Trends Immunol.* **2017**, *38*, 526–536. [CrossRef] [PubMed]

178. Gerber, S.A.; Rybalko, V.Y.; Bigelow, C.E.; Lugade, A.A.; Foster, T.H.; Frelinger, J.G.; Lord, E.M. Preferential attachment of peritoneal tumor metastases to omental immune aggregates and possible role of a unique vascular microenvironment in metastatic survival and growth. *Am. J. Pathol.* **2006**, *169*, 1739–1752. [CrossRef] [PubMed]

179. Clark, R.; Krishnan, V.; Schoof, M.; Rodriguez, I.; Theriault, B.; Chekmareva, M.; Rinker-Schaeffer, C. Milky spots promote ovarian cancer metastatic colonization of peritoneal adipose in experimental models. *Am. J. Pathol.* **2013**, *183*, 576–591. [CrossRef] [PubMed]

180. Szatrowski, T.P.; Nathan, C.F. Production of large amounts of hydrogen peroxide by human tumor cells. *Cancer Res.* **1991**, *51*, 794–798. [PubMed]

181. Wang, Y.; Ma, J.; Shen, H.; Wang, C.; Sun, Y.; Howell, S.B.; Lin, X. Reactive oxygen species promote ovarian cancer progression via the HIF-1α/LOX/E-cadherin pathway. *Oncol. Rep.* **2014**, *32*, 2150–2158. [CrossRef] [PubMed]

182. Kundu, N.; Zhang, S.; Fulton, A.M. Sublethal oxidative stress inhibits tumor cell adhesion and enhances experimental metastasis of murine mammary carcinoma. *Clin. Exp. Metast.* **1995**, *13*, 16–22. [CrossRef]

183. Haskill, S.; Becker, S.; Fowler, W.; Walton, L. Mononuclear-cell infiltration in ovarian cancer. I. Inflammatory-cell infiltrates from tumour and ascites material. *Br. J. Cancer* **1982**, *45*, 728–736. [CrossRef] [PubMed]

184. Wang, J.M.; Griffin, J.D.; Rambaldi, A.; Chen, Z.G.; Mantovani, A. Induction of monocyte migration by recombinant macrophage colony-stimulating factor. *J. Immunol.* **1988**, *141*, 575–579. [PubMed]

185. Ramakrishnan, S.; Xu, F.J.; Brandt, S.J.; Niedel, J.E.; Bast, R.C., Jr.; Brown, E.L. Constitutive production of macrophage colony-stimulating factor by human ovarian and breast cancer cell lines. *J. Clin. Investig.* **1989**, *83*, 921–926. [CrossRef] [PubMed]

186. Plante, M.; Rubin, S.C.; Wong, G.Y.; Federici, M.G.; Finstad, C.L.; Gastl, G.A. Interleukin-6 level in serum and ascites as a prognostic factor in patients with epithelial ovarian cancer. *Cancer* **1994**, *73*, 1882–1888. [CrossRef]

187. Scambia, G.; Testa, U.; Panici, P.B.; Martucci, R.; Foti, E.; Petrini, M.; Amoroso, M.; Masciullo, V.; Peschle, C.; Mancuso, S. Interleukin-6 serum levels in patients with gynecological tumors. *Int. J. Cancer* **1994**, *57*, 318–323. [CrossRef] [PubMed]

188. Tempfer, C.; Zeisler, H.; Sliutz, G.; Haeusler, G.; Hanzal, E.; Kainz, C. Serum evaluation of interleukin 6 in ovarian cancer patients. *Gynecol. Oncol.* **1997**, *66*, 27–30. [CrossRef] [PubMed]

189. Isobe, A.; Sawada, K.; Kinose, Y.; Ohyagi-Hara, C.; Nakatsuka, E.; Makino, H.; Ogura, T.; Mizuno, T.; Suzuki, N.; Morii, E.; et al. Interleukin 6 receptor is an independent prognostic factor and a potential therapeutic target of ovarian cancer. *PLoS ONE* **2015**, *10*, e0118080. [CrossRef] [PubMed]

190. Fishman, D.A.; Liu, Y.; Ellerbroek, S.M.; Stack, M.S. Lysophosphatidic acid promotes matrix metalloproteinase (MMP) activation and MMP-dependent invasion in ovarian cancer cells. *Cancer Res.* **2001**, *61*, 3194–3199. [PubMed]

191. Reiser, C.O.; Lanz, T.; Hofmann, F.; Hofer, G.; Rupprecht, H.D.; Goppelt-Struebe, M. Lysophosphatidic acid-mediated signal-transduction pathways involved in the induction of the early-response genes prostaglandin G/H synthase-2 and Egr-1: A critical role for the mitogen-activated protein kinase p38 and for Rho proteins. *Biochem. J.* **1998**, *330 (Pt 3)*, 1107–1114. [CrossRef] [PubMed]

192. Moolenaar, W.H.; Kruijer, W.; Tilly, B.C.; Verlaan, I.; Bierman, A.J.; de Laat, S.W. Growth factor-like action of phosphatidic acid. *Nature* **1986**, *323*, 171–173. [CrossRef] [PubMed]

193. Schwartz, B.M.; Hong, G.; Morrison, B.H.; Wu, W.; Baudhuin, L.M.; Xiao, Y.J.; Mok, S.C.; Xu, Y. Lysophospholipids increase interleukin-8 expression in ovarian cancer cells. *Gynecol. Oncol.* **2001**, *81*, 291–300. [CrossRef] [PubMed]

194. Yu, X.; Zhang, Y.; Chen, H. LPA receptor 1 mediates LPA-induced ovarian cancer metastasis: An in vitro and in vivo study. *BMC Cancer* **2016**, *16*, 846. [CrossRef] [PubMed]

195. Loizzi, V.; Del Vecchio, V.; Gargano, G.; De Liso, M.; Kardashi, A.; Naglieri, E.; Resta, L.; Cicinelli, E.; Cormio, G. Biological Pathways Involved in Tumor Angiogenesis and Bevacizumab Based Anti-Angiogenic Therapy with Special References to Ovarian Cancer. *Int. J. Mol. Sci.* **2017**, *18*, 1967. [CrossRef] [PubMed]

196. Le Page, C.; Puiffe, M.L.; Meunier, L.; Zietarska, M.; de Ladurantaye, M.; Tonin, P.N.; Provencher, D.; Mes-Masson, A.M. BMP-2 signaling in ovarian cancer and its association with poor prognosis. *J. Ovarian Res.* **2009**, *2*, 4. [CrossRef] [PubMed]

197. Ozols, R.F. Treatment goals in ovarian cancer. *Int. J. Gynecol. Cancer* **2005**, *15* (Suppl. 1), 3–11. [CrossRef] [PubMed]

198. Koti, M.; Siu, A.; Clement, I.; Bidarimath, M.; Turashvili, G.; Edwards, A.; Rahimi, K.; Mes-Masson, A.M.; Squire, J.A. A distinct pre-existing inflammatory tumour microenvironment is associated with chemotherapy resistance in high-grade serous epithelial ovarian cancer. *Br. J. Cancer* **2015**, *112*, 1215–1222. [CrossRef] [PubMed]

199. Stewart, D.J. Mechanisms of resistance to cisplatin and carboplatin. *Crit Rev. Oncol Hematol* **2007**, *63*, 12–31. [CrossRef] [PubMed]

200. Damiano, J.S.; Cress, A.E.; Hazlehurst, L.A.; Shtil, A.A.; Dalton, W.S. Cell adhesion mediated drug resistance (CAM-DR): Role of integrins and resistance to apoptosis in human myeloma cell lines. *Blood* **1999**, *93*, 1658–1667. [PubMed]

201. Meads, M.B.; Hazlehurst, L.A.; Dalton, W.S. The bone marrow microenvironment as a tumor sanctuary and contributor to drug resistance. *Clin. Cancer Res.* **2008**, *14*, 2519–2526. [CrossRef] [PubMed]

202. Catlett-Falcone, R.; Landowski, T.H.; Oshiro, M.M.; Turkson, J.; Levitzki, A.; Savino, R.; Ciliberto, G.; Moscinski, L.; Fernandez-Luna, J.L.; Nunez, G.; et al. Constitutive activation of Stat3 signaling confers resistance to apoptosis in human U266 myeloma cells. *Immunity* **1999**, *10*, 105–115. [CrossRef]

203. Frassanito, M.A.; Cusmai, A.; Iodice, G.; Dammacco, F. Autocrine interleukin-6 production and highly malignant multiple myeloma: Relation with resistance to drug-induced apoptosis. *Blood* **2001**, *97*, 483–489. [CrossRef] [PubMed]

204. Meads, M.B.; Gatenby, R.A.; Dalton, W.S. Environment-mediated drug resistance: A major contributor to minimal residual disease. *Nat. Rev. Cancer* **2009**, *9*, 665–674. [CrossRef] [PubMed]

205. Cohen, S.; Mehrabi, S.; Yao, X.; Millingen, S.; Aikhionbare, F.O. Reactive Oxygen Species and Serous Epithelial Ovarian Adenocarcinoma. *Cancer Res. J.* **2016**, *4*, 106–114. [CrossRef] [PubMed]

206. St-Pierre, J.; Drori, S.; Uldry, M.; Silvaggi, J.M.; Rhee, J.; Jager, S.; Handschin, C.; Zheng, K.; Lin, J.; Yang, W.; et al. Suppression of reactive oxygen species and neurodegeneration by the PGC-1 transcriptional coactivators. *Cell* **2006**, *127*, 397–408. [CrossRef] [PubMed]

207. Kim, B.; Jung, J.W.; Jung, J.; Han, Y.; Suh, D.H.; Kim, H.S.; Dhanasekaran, D.N.; Song, Y.S. PGC1α induced by reactive oxygen species contributes to chemoresistance of ovarian cancer cells. *Oncotarget* **2017**, *8*, 60299–60311. [PubMed]

208. Duan, Z.; Feller, A.J.; Penson, R.T.; Chabner, B.A.; Seiden, M.V. Discovery of differentially expressed genes associated with paclitaxel resistance using cDNA array technology: Analysis of interleukin (IL) 6, IL-8, and monocyte chemotactic protein 1 in the paclitaxel-resistant phenotype. *Clin. Cancer Res.* **1999**, *5*, 3445–3453. [PubMed]

209. Duan, Z.; Foster, R.; Bell, D.A.; Mahoney, J.; Wolak, K.; Vaidya, A.; Hampel, C.; Lee, H.; Seiden, M.V. Signal transducers and activators of transcription 3 pathway activation in drug-resistant ovarian cancer. *Clin. Cancer Res.* **2006**, *12*, 5055–5063. [CrossRef] [PubMed]

210. Abdollahi, T.; Robertson, N.M.; Abdollahi, A.; Litwack, G. Identification of interleukin 8 as an inhibitor of tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis in the ovarian carcinoma cell line OVCAR3. *Cancer Res.* **2003**, *63*, 4521–4526. [PubMed]

211. Wiley, S.R.; Schooley, K.; Smolak, P.J.; Din, W.S.; Huang, C.P.; Nicholl, J.K.; Sutherland, G.R.; Smith, T.D.; Rauch, C.; Smith, C.A.; et al. Identification and characterization of a new member of the TNF family that induces apoptosis. *Immunity* **1995**, *3*, 673–682. [CrossRef]

212. Walczak, H.; Miller, R.E.; Ariail, K.; Gliniak, B.; Griffith, T.S.; Kubin, M.; Chin, W.; Jones, J.; Woodward, A.; Le, T.; et al. Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand in vivo. *Nat. Med.* **1999**, *5*, 157–163. [CrossRef] [PubMed]

213. Bristow, R.E.; Baldwin, R.L.; Yamada, S.D.; Korc, M.; Karlan, B.Y. Altered expression of transforming growth factor-beta ligands and receptors in primary and recurrent ovarian carcinoma. *Cancer* **1999**, *85*, 658–668. [CrossRef]

214. Li, M.; Balch, C.; Montgomery, J.S.; Jeong, M.; Chung, J.H.; Yan, P.; Huang, T.H.; Kim, S.; Nephew, K.P. Integrated analysis of DNA methylation and gene expression reveals specific signaling pathways associated with platinum resistance in ovarian cancer. *BMC Med. Genom.* **2009**, *2*, 34. [CrossRef] [PubMed]

215. Psyrri, A.; Kassar, M.; Yu, Z.; Bamias, A.; Weinberger, P.M.; Markakis, S.; Kowalski, D.; Camp, R.L.; Rimm, D.L.; Dimopoulos, M.A. Effect of epidermal growth factor receptor expression level on survival in patients with epithelial ovarian cancer. *Clin. Cancer Res.* **2005**, *11*, 8637–8643. [CrossRef] [PubMed]

216. Crew, A.J.; Langdon, S.P.; Miller, E.P.; Miller, W.R. Mitogenic effects of epidermal growth factor and transforming growth factor-α on EGF-receptor positive human ovarian carcinoma cell lines. *Eur. J. Cancer* **1992**, *28*, 337–341. [CrossRef]

217. Ferrandina, G.; Ranelletti, F.O.; Martinelli, E.; Paglia, A.; Zannoni, G.F.; Scambia, G. Cyclo-oxygenase-2 (Cox-2) expression and resistance to platinum versus platinum/paclitaxel containing chemotherapy in advanced ovarian cancer. *BMC Cancer* **2006**, *6*, 182. [CrossRef] [PubMed]

218. Ferrandina, G.; Lauriola, L.; Zannoni, G.F.; Fagotti, A.; Fanfani, F.; Legge, F.; Maggiano, N.; Gessi, M.; Mancuso, S.; Ranelletti, F.O.; et al. Increased cyclooxygenase-2 (COX-2) expression is associated with chemotherapy resistance and outcome in ovarian cancer patients. *Ann. Oncol.* **2002**, *13*, 1205–1211. [CrossRef] [PubMed]

219. Kim, A.; Ueda, Y.; Naka, T.; Enomoto, T. Therapeutic strategies in epithelial ovarian cancer. *J. Exp. Clin. Cancer Res.* **2012**, *31*, 14. [CrossRef] [PubMed]

220. Ellis, L.M.; Hicklin, D.J. VEGF-targeted therapy: Mechanisms of anti-tumour activity. *Nat. Rev. Cancer* **2008**, *8*, 579–591. [CrossRef] [PubMed]

221. Aghajanian, C.; Blank, S.V.; Goff, B.A.; Judson, P.L.; Teneriello, M.G.; Husain, A.; Sovak, M.A.; Yi, J.; Nycum, L.R. OCEANS: A randomized, double-blind, placebo-controlled phase III trial of chemotherapy with or without bevacizumab in patients with platinum-sensitive recurrent epithelial ovarian, primary peritoneal, or fallopian tube cancer. *J. Clin. Oncol.* **2012**, *30*, 2039–2045. [CrossRef] [PubMed]

222. Burger, R.A.; Brady, M.F.; Bookman, M.A.; Fleming, G.F.; Monk, B.J.; Huang, H.; Mannel, R.S.; Homesley, H.D.; Fowler, J.; Greer, B.E.; et al. Incorporation of bevacizumab in the primary treatment of ovarian cancer. *N. Engl. J. Med.* **2011**, *365*, 2473–2483. [CrossRef] [PubMed]

223. Perren, T.J.; Swart, A.M.; Pfisterer, J.; Ledermann, J.A.; Pujade-Lauraine, E.; Kristensen, G.; Carey, M.S.; Beale, P.; Cervantes, A.; Kurzeder, C.; et al. A phase 3 trial of bevacizumab in ovarian cancer. *N. Engl. J. Med.* **2011**, *365*, 2484–2496. [CrossRef] [PubMed]

224. Brown, E.R.; Charles, K.A.; Hoare, S.A.; Rye, R.L.; Jodrell, D.I.; Aird, R.E.; Vora, R.; Prabhakar, U.; Nakada, M.; Corringham, R.E.; et al. A clinical study assessing the tolerability and biological effects of infliximab, a TNF-α inhibitor, in patients with advanced cancer. *Ann. Oncol.* **2008**, *19*, 1340–1346. [CrossRef] [PubMed]

225. Guo, Y.; Nemeth, J.; O'Brien, C.; Susa, M.; Liu, X.; Zhang, Z.; Choy, E.; Mankin, H.; Hornicek, F.; Duan, Z. Effects of siltuximab on the IL-6-induced signaling pathway in ovarian cancer. *Clin. Cancer Res.* **2010**, *16*, 5759–5769. [CrossRef] [PubMed]

226. Xu, S.; Grande, F.; Garofalo, A.; Neamati, N. Discovery of a novel orally active small-molecule gp130 inhibitor for the treatment of ovarian cancer. *Mol. Cancer Ther.* **2013**, *12*, 937–949. [CrossRef] [PubMed]

227. Dijkgraaf, E.M.; Santegoets, S.J.; Reyners, A.K.; Goedemans, R.; Wouters, M.C.; Kenter, G.G.; van Erkel, A.R.; van Poelgeest, M.I.; Nijman, H.W.; van der Hoeven, J.J.; et al. A phase I trial combining carboplatin/doxorubicin with tocilizumab, an anti-IL-6R monoclonal antibody, and interferon-α2b in patients with recurrent epithelial ovarian cancer. *Ann. Oncol.* **2015**, *26*, 2141–2149. [CrossRef] [PubMed]

228. Bhola, N.E.; Balko, J.M.; Dugger, T.C.; Kuba, M.G.; Sanchez, V.; Sanders, M.; Stanford, J.; Cook, R.S.; Arteaga, C.L. TGF-beta inhibition enhances chemotherapy action against triple-negative breast cancer. *J. Clin. Investig.* **2013**, *123*, 1348–1358. [CrossRef] [PubMed]

229. Alsina-Sanchis, E.; Figueras, A.; Lahiguera, A.; Gil-Martin, M.; Pardo, B.; Piulats, J.M.; Marti, L.; Ponce, J.; Matias-Guiu, X.; Vidal, A.; et al. TGFbeta Controls Ovarian Cancer Cell Proliferation. *Int. J. Mol. Sci.* **2017**, *18*, 1658. [CrossRef] [PubMed]

230. Secord, A.A.; Blessing, J.A.; Armstrong, D.K.; Rodgers, W.H.; Miner, Z.; Barnes, M.N.; Lewandowski, G.; Mannel, R.S.; Gynecologic Oncology, G. Phase II trial of cetuximab and carboplatin in relapsed platinum-sensitive ovarian cancer and evaluation of epidermal growth factor receptor expression: A Gynecologic Oncology Group study. *Gynecol. Oncol.* **2008**, *108*, 493–499. [CrossRef] [PubMed]

231. Chekerov, R.; Klare, P.; Krabisch, P.; Potenberg, J.; Heinrich, G.; Mueller, L.; Kurbacher, C.M.; Grischke, E.-M.; Braicu, E.I.; Wimberger, P.; et al. Panitumumab in platinum-sensitive epithelial ovarian cancer patients with KRAS wild-type: The PROVE-study, a phase II randomized multicenter study of the North-Eastern German Society of Gynaecologic Oncology. *J. Clin. Oncol.* **2017**, *35* (Suppl. 1), 5558.

Exhibit 77

REVIEW

Possible Role of Ovarian Epithelial Inflammation in Ovarian Cancer

Roberta B. Ness, Carrie Cottreau

Ovarian cancer is a commonly fatal disease for which prevention strategies have been limited, in part because of a lack of understanding of the underlying biology. This paper reviews the epidemiologic literature in the English language on risk factors and protective factors for ovarian cancer and proposes a novel hypothesis that a common mechanism underlying this disease is inflammation. Previous hypotheses about the causes of ovarian cancer have attributed risk to an excess number of lifetime ovulations or to elevations in steroid hormones. Inflammation may underlie ovulatory events because an inflammatory reaction is induced during the process of ovulation. Additional risk factors for ovarian cancer, including asbestos and talc exposure, endometriosis (i.e., ectopic implantation of uterine lining tissue), and pelvic inflammatory disease, cannot be directly linked to ovulation or to hormones but do cause local pelvic inflammation. On the other hand, tubal ligation and hysterectomy act as protective factors, perhaps by diminishing the likelihood that the ovarian epithelium will be exposed to environmental initiators of inflammation. Inflammation entails cell damage, oxidative stress, and elevations of cytokines and prostaglandins, all of which may be mutagenic. The possibility that inflammation is a pathophysiologic contributor to the development of ovarian cancer suggests a directed approach to future research [J Natl Cancer Inst 1999;91:1459–67]

Ovarian cancer is the gynecologic cancer most likely to result in death among women *(1),* yet the pathophysiology underlying epithelial ovarian cancer is not clearly established. For many years, two dominant hypotheses—the ovulation hypothesis *(2–4),* which relates ovarian cancer risk to incessant ovulation, and the pituitary gonadotropin hypothesis *(5),* which implicates elevations in gonadotropin levels acting in concert with estrogen—have sought to explain the genesis of this disease. Epidemiologic and biologic data have not been entirely consistent with either of these hypotheses. At the same time, a growing body of epidemiologic evidence suggests that factors causing epithelial inflammation are involved in ovarian carcinogenesis. Such factors include asbestos and talc exposures, endometriosis, and pelvic inflammatory disease (PID). Conversely, there appear to be protective effects of tubal ligation and hysterectomy, which may reduce the exposure from local genital tract irritants. We first briefly review evidence for and against the ovulation and gonadotropin hypotheses. We then propose that inflammation may work in conjunction with, and in addition to, ovulation and steroid hormones in mediating epithelial ovarian cancer risk (Fig. 1).

In this review, only epithelial ovarian cancers will be discussed because they account for about 90% of all ovarian cancers. We will not discriminate between invasive and noninvasive tumors, since both have similar risk factors. Also, we acknowledge the potential heterogeneity between mucinous and other epithelial ovarian tumor types *(6,7),* but histology-specific considerations are beyond the scope of this review.

Studies were identified for this review by searching the English language literature in the MEDLINE® database and by an extensive review of bibliographies from articles found through that search.

EVIDENCE SUPPORTING THE PITUITARY GONADOTROPIN AND OVULATION HYPOTHESES

The factors that afford the greatest overall risk reduction for ovarian cancer in female populations are parity (number of live births) *(6,8–36),* oral contraceptive use *(6,8–16,24,31,32,35–45),* and prolonged breast-feeding *(31,46).* During pregnancy, very high levels of estrogen and progesterone suppress levels of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) and disallow ovulation; during oral contraceptive use, stable levels of estrogens and progestins inhibit the gonadotropins and their ability to stimulate ovulation; and during breast-feeding, low levels of estrogen and LH suppress ovulation *(47).* That these reproductive and contraceptive factors are protective suggests a common effect through ovulation or steroid hormones. Oral contraceptive use, parity, and breast-feeding each provide a reduction in risk for two to three decades after their cessation, so that they must trigger biologic events that do not clinically manifest themselves as cancer until many years thereafter *(48).*

If fertility drugs were found to influence the development of ovarian cancer, this influence would also potentially support both the ovulation and gonadotropin hypotheses, since these drugs both elevate gonadotropin levels and cause superovulation. However, the literature *(49,50)* is conflicting regarding the association between the use of fertility drugs and ovarian cancer.

SCRUTINIZING THE PITUITARY GONADOTROPIN HYPOTHESIS

The pituitary gonadotropin hypothesis suggests that critical events in the transformation to ovarian cancer are the entrapment

---

Affiliations of authors: R. B. Ness, Department of Epidemiology, University of Pittsburgh Graduate School of Public Health, University of Pittsburgh Cancer Institute, and Magee-Womens Research Institute, Pittsburgh, PA; C. Cottreau, Department of Epidemiology, University of Pittsburgh Graduate School of Public Health.

Correspondence to: Roberta B. Ness, M.D., M.P.H., University of Pittsburgh Graduate School of Public Health, 130 DeSoto St., Rm. 513, Parran Hall, Pittsburgh, PA 15261 (e-mail: repro@vms.cis.pitt.edu).

See "Note" following "References."

```
┌─────────────────────────────────────────────────────────────────┐
│  ┌──────────────────────┐        ┌──────────────────────────┐    │
│  │ Epithelial inflammation│        │ Persistently high levels │    │
│  │ initiators            │        │ of steroid hormones,     │    │
│  └──────────────────────┘        │ gonadotrophins           │    │
│          ⊕ ↘                      └──────────────────────────┘    │
│                                        ↙ ⊕                        │
│  ┌────────────────┐  ⊖  ┌─────────────────────┐  ⊕  ┌──────────┐ │
│  │ Hysterectomy and│ →   │ INFLAMMATION        │  ←  │ Ovulation│ │
│  │ tubal ligation  │     │ - DNA damage and    │     └──────────┘ │
│  └────────────────┘     │   repair            │                   │
│                         │ - Oxidative stress  │                   │
│                         │ - Elevated cytokines│                   │
│                         │   and prostaglandins│                   │
│                         └─────────────────────┘                   │
│                              ↓                                    │
│                    ┌──────────────────────┐                       │
│                    │ OVARIAN CARCINOGENESIS│                      │
│                    └──────────────────────┘                       │
└─────────────────────────────────────────────────────────────────┘
```
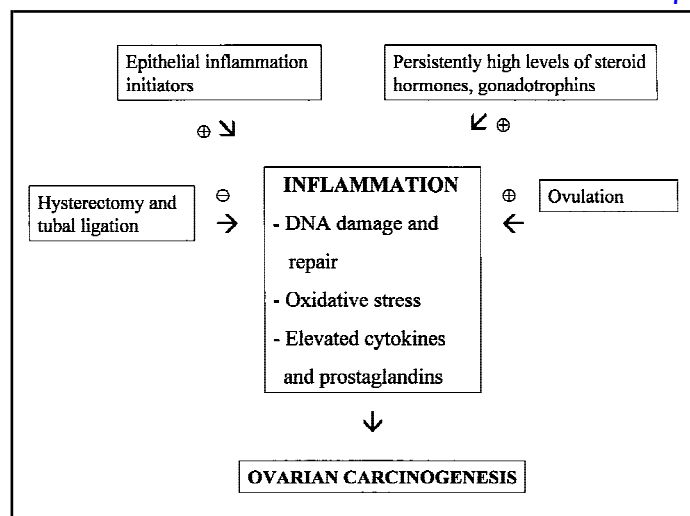
Fig. 1. Inflammation as a common mechanism underlying ovarian cancer.

of surface epithelium in inclusion cysts followed by stimulation of the entrapped epithelium by estrogen or estrogen precursors, particularly in the presence of high and persistent levels of gonadotropins (LH and FSH) (5). Several observations do not completely fit the pituitary gonadotropin hypothesis. High estrogen levels alone could not be the whole story behind mutagenicity because estrogen levels are at their highest during pregnancy, a reproductive event that is strongly protective for ovarian cancer (48). In addition, one study (51) found no estrogen receptors in epithelium on the surface of the ovary or in inclusion cysts. Cramer and Welch (5) illustrated the nature of the proposed interplay between gonadotropins and estrogens and suggested that disruption of negative feedback to the pituitary in the presence of an otherwise normal ovarian steroidal environment (e.g., by transplanting the ovary to the spleen wherein ovarian hormones would be degraded by the liver) would elevate gonadotropins and stimulate ovarian mutagenesis. A pharmacologic equivalent to this would be use of medications, such as barbiturates, halogenated hydrocarbon pesticides, anti-inflammatory medications, and antihistamines, that would degrade estrogen at a greater than normal rate. However, to our knowledge, there has not been any evidence that such medications increase the risk of ovarian cancer (52). These authors (5,53) also proposed that premature ovarian failure or early menopause could be associated with elevated ovarian cancer risk via high gonadotropin levels. However, there is little evidence that age at natural menopause influences risk (32,46).

Furthermore, in the only prospective study to examine this question directly (54), gonadotropin levels measured from serum stored many years prior to outcome were not associated with the occurrence of ovarian cancer. Helzlsouer et al. (54) analyzed levels of LH, FSH, and other hormones among case patients with ovarian cancer and control subjects arising from a prospective population-based serum bank study. Of 20 305 participants from whom serum had been collected and frozen, 31 who were not taking hormone replacement therapy (HRT) at baseline developed ovarian cancer a mean of 8 years after blood collection. These case patients were matched to 62 control subjects on age, menopausal status, and, for premenopausal women, number of days from the beginning of the last menstrual period. Mean levels of FSH, LH, and estrogens were somewhat lower among

case patients with ovarian cancer than among control subjects, whereas the androgens androstenedione, dihydroepiandosterone, and dihydroepiandosterone sulfate were associated with an increased risk. These results do not support the hypothesis that elevated pituitary gonadotropin levels increase ovarian cancer risk. However, limitations of the study were the measurement of hormones at a single point in time, the inclusion of premenopausal women without precise determination of timing of blood collection within the menstrual cycle, the small number of cases of ovarian cancer, and the limited adjustment for confounding factors.

A more complex issue that is somewhat difficult to reconcile with the gonadotropin hypothesis is that postmenopausal estrogen use has been modestly, albeit inconsistently, associated with increased risk for ovarian cancer (7,8,11,12,14,18,24,36,55–62). A recent meta-analysis (63), including 11 articles with data from 21 studies, did show a small increase in overall risk with HRT use (relative risk [RR] = 1.15; 95% confidence interval [CI] = 1.05–1.27) with a somewhat higher risk, albeit of borderline significance, among users for more than 10 years' duration (RR = 1.27; 95% CI = 1.00–1.61). Rodriguez et al. (62) in the prospective Nurses Health Study found 18 cases in 5000 person-years among long-term users (>11 years), for an RR of 1.7 (95% CI = 1.1–2.8). Postmenopausal estrogens reduce gonadotropins and increase estrogen levels. To the degree that the gonadotropin hypothesis predicted that excess LH and FSH stimulate mutagenesis, these findings would seem to counter the predictions of the hypothesis. However, if the hormonal mechanism more relevant to the thesis of the gonadotropin hypothesis were that of estrogen elevation, then these findings would indeed fit the data. Taken together, the literature reviewed above does not fully support the gonadotropin hypothesis, although it is quite possible that steroid hormones do play some role in pathogenesis.

SCRUTINIZING THE OVULATION HYPOTHESIS

The ovulation hypothesis states that excessive ovulation damages the ovarian epithelium, from which epithelial ovarian cancer arises (2). This hypothesis proposes that repeated cell damage translates into an enhanced potential for aberrant DNA repair, inactivation of tumor-suppressor genes, and subsequent mutagenesis (3,4). Perhaps the most complex issue to reconcile with the ovulation hypothesis is whether ovulatory infertility increases the risk for ovarian cancer. Ovulatory infertility is the result of a lack of ovulation and so should not elevate the risk of ovarian cancer according to this hypothesis. Although several studies [reviewed at length elsewhere (49)] have shown that ovarian cancer is associated with difficulty in achieving pregnancy (8,21,31,64–69), there has been inconsistency regarding the type of infertility associated with risk. With regard to ovulatory infertility, Rossing et al. (64) examined records of women who presented to infertility clinics in Seattle, WA, during the period from 1974 through 1985 and who were subsequently identified through cancer registry information if they developed ovarian cancer. Based on small numbers, the RR for ovulatory abnormalities was 3.7 (95% CI = 1.4–8.1) when compared with population-based expected rates of ovarian cancer. This analysis was limited by the likelihood that the external comparison population would likely be more parous, more likely to have used oral contraceptives, and therefore at a lower ovarian cancer risk—hence, resulting in an inflation of the observed RR. In fact, when Rossing et al. compared women with ovulatory infertility with

internal control subjects who had other infertility diagnoses, the risk of ovarian cancer was 1.8 (95% CI = 0.5–6.1). Venn et al. *(65)* published data from a larger retrospective cohort study of women attending an *in vitro* fertilization clinic and compared their rates with population-based ovarian cancer rates. Again, infertility was associated with ovarian cancer, but only for women with unexplained infertility (odds ratio [OR] = 19.2; 95% CI = 2.2–165) and not for women with ovulatory infertility. In summary, because anovulation is only one among several possible causes of infertility, this limited literature neither supports nor refutes the ovulation hypothesis.

Factors that reduce ovulation do not proportionally reduce the risk of ovarian cancer *(24,46)*. First proposed by Risch et al. *(24)* and later demonstrated by Whittemore et al. *(46)*, 1 year of delayed menarche or of early menopause was associated with a much less marked reduction in ovarian cancer risk than was 1 year of term pregnancy, 1 year of breast-feeding, or 1 year of oral contraceptive use. Were the ovulation hypothesis to hold, there is no reason to imagine that various sources of ovulation cessation would differentially impact risk. However, age at menarche and age at menopause may less accurately reflect ovulatory function than does pregnancy or oral contraceptive use; the initiation and cessation of menses do not reflect the initiation and cessation of ovulation *(70)*. Nevertheless, suppression of ovulation cannot fully account for the risk reductions observed in epidemiologic studies. Assuming that ovulations occur over a period of at least 20 years, a full-term pregnancy would be expected to reduce ovarian cancer risk by 5%, whereas Whittemore et al. *(46)* observed about a 15% reduction in risk for each pregnancy after the first.

EPIDEMIOLOGIC DATA SUPPORTING THE ROLE OF LOCAL INFLAMMATION IN OVARIAN CANCER RISK

Several types of exposure that do not directly affect ovulation or steroid hormone levels but that do enhance local inflammation have been implicated as ovarian cancer risk factors. Reduced passage of inflammatory toxins from the lower to the upper genital tract may also reduce risk.

TALC AND ASBESTOS EXPOSURE

In the early 1960s, it was recognized that female asbestos workers had an increased risk of developing ovarian cancer and other intra-abdominal neoplasms *(71,72)*. Subsequent retrospective cohort studies of women who were employed in industries wherein they might encounter heavy asbestos exposure *(73–75)* found about a twofold excess of ovarian cancers over what was expected, with a dose–response relationship suggested. Heller et al. *(76)* documented that substantial amounts of asbestos fiber could be detected in the ovarian tissues of women whose fathers or husbands worked in occupations in which asbestos exposure was high. The rates of finding asbestos in ovarian tissue were twice as high in women with household exposure as in women without such an exposure history. Animal models *(73,77,78)* provide some support for the suggestion that asbestos exposure may cause ovarian cancer. Intraperitoneal injection of asbestos into guinea pigs and rabbits results in changes in the ovarian epithelium similar to those seen in early ovarian cancer in women; similar changes were found among 20% of the exposed and 0% of the unexposed animals *(77)*. However, whereas asbestos was cytotoxic to hamster ovary cells *in vitro (78)*, it had no effect on the ovaries of mice and hamsters *in vivo (77)*.

Although household-related asbestos exposure may be related to dust on the clothing, with those who launder the clothing at increased risk of cancer, it is also possible that exposure occurs through sexual intercourse with particles traveling from the lower to the upper genital tract. Traffic of endogenous cells and pathogens from the lower to the upper genital tract has been shown to be common *(79)*. This fact links cervicitis, i.e., sexually transmitted infection of the lower genital tract epithelium, to PID. It may also link asbestos exposure and talc use to ovarian epithelial inflammation.

Talc, which is structurally similar to asbestos, has repeatedly been related to ovarian cancer. Prior to 1976, talc was commonly contaminated with asbestos, so that the early studies relating talc to ovarian cancer may have been confounded by the asbestos–ovarian cancer relationship *(80)*. More recent findings are less likely to be solely driven by the asbestos relationship.

At least 12 epidemiologic studies *(8,81–91)* have evaluated the use of talc in relationship to ovarian cancer. Eight of these studies *(81–87,90)* reported an elevated cancer risk among women whose powder exposure was described as a "dusting of the perineum," with ORs ranging from 1.3 to 3.9. Two other studies *(8,88)* found a very small elevation in risk with the use of a more general exposure definition, and one study *(89)* found no association. In the most extensive and focused analysis to date, Cook et al. *(81)* interviewed 313 case patients with ovarian cancer and 422 control subjects regarding exposure to a variety of powder products used in a series of ways (e.g., perineal dusting, diaphragm storage, powder on sanitary napkins, and genital deodorant spray). Both talc-containing and non-talc-containing baby or bath powder products were associated with an elevated risk of ovarian cancer; each way of using it, with the exception of diaphragm storage, was also associated with an elevated risk of ovarian cancer. A limited number of studies *(8,81,90,92)* have examined the potential for a dose–response relationship. Some studies have shown some increase in risk with more frequent exposure *(83,86)*, longer exposure *(86)*, and greater total number of lifetime applications *(86)*. However, other studies *(8,81,90)* have not shown any dose–response relationship. The link between talc exposure and ovarian cancer is limited by a lack of supportive animal data and an inconsistency in the detection of talc in the ovarian tissue of women who reported heavy use *(91)*. Nevertheless, the consistency of an association between talc use and ovarian cancer in a series of well-conducted studies of varying design suggests that talc use may represent another environmental exposure that enhances epithelial inflammation and thereby either initiates or promotes ovarian carcinogenesis.

ENDOMETRIOSIS

Endometriosis is the presence of endometrial tissue outside the lining of the uterus. Although the cause of endometriosis is unknown, it is clear that the implantation of ectopic endometrial tissue is associated with a local inflammatory reaction, including macrophage activation, and elevation of cytokines and growth factors.

Ovarian tumors arise out of ovarian endometriosis in 0.3%–0.8% of case patients who are followed clinically *(93,94)*. In the most extensive epidemiologic study to date, Brinton et al. *(95)* assessed cancer outcomes among 20 686 women with endometriosis who were hospitalized in Sweden. Hospitalizations were identified through the nationwide Swedish Inpatient Registrar, and outcomes were identified through the National Swedish

Cancer Registry after a mean of 11.4 years of follow-up. The risk of ovarian cancer was elevated 2.5-fold for women followed for 10 or more years, and the risk rose to more than fourfold among women whose endometriosis was located in the ovaries. Unfortunately, this study did not control for parity or oral contraceptive use, which might have led to an inflated estimate of risk. However, there is also substantial clinical support for an association between endometriosis and ovarian cancer.

Several case series (93,96–101) have demonstrated cancer tumorigenesis that arises from endometriosis. Sampson (102), who documented the first case, outlined a set of criteria for establishing the existence of such a cancerous transformation. These criteria include the following: 1) demonstration of both cancerous and benign endometrial tissues in the same ovary, 2) demonstration of cancer arising in the tissue and not invading from another source, and 3) demonstration of a histologic relationship between invasive and benign components. Reviewing the literature, Heaps et al. (93) noted that 165 cases have been published that meet these criteria. Almost 80% of these malignant transformations arose from ovarian endometriosis, and the rest came from extragonadal sites. Endometrioid adenocarcinomas accounted for 69% of lesions, followed by clear-cell carcinomas (13.5%) and sarcomas (11.6%). This is a far higher proportion of endometrioid and clear-cell tumors than is found among ovarian cancers in general (10%–20% and 3%–10%, respectively), which again points to a possible transformation from endometriosis to specific types of endometrial cancer (103). One case report (99) documented the experience of a woman who, on biopsy, first showed atypia within ovarian endometriosis and then 3 years later had a clear-cell ovarian carcinoma arising from the same ovary. Finally, Sainz de la Cuesta et al. (96) found endometriosis among about 40% of women with stage I endometrioid or clear-cell ovarian carcinoma, about one third of which were carcinomas arising out of endometriosis. Czernobilsky and Morris (104) also showed that mild cytologic atypia occurred in about 20% of endometriosis lesions and that severe atypia, a probable precursor of ovarian cancer, occurred in 3.6%. Taken as a whole, these data strongly support a temporal pattern of transition from simple endometriosis to atypical endometriosis to ovarian cancer.

HYSTERECTOMY AND BILATERAL TUBAL LIGATION

Hysterectomy without oophorectomy and tubal ligation both have been associated with reductions in the risk for ovarian cancer (105–115). ORs have ranged from 0.03 to 0.8 for hysterectomy and from 0.2 to 0.9 for tubal ligation. Some authors (105–107) found that the protective effect for hysterectomy waned after 5–20 years and suggested that the observed protection afforded by these procedures might result from screening whereby ovaries examined at the time of surgery and found to be abnormal were removed. However, other authors (6,108,114) found that the protection afforded by hysterectomy or tubal ligation continues for 20–25 years after the procedure. Green et al. (114) proposed that the mechanism whereby hysterectomy and tubal ligation protect against ovarian cancer is by cutting off the pathway between the lower and the upper parts of the genital tract, thereby disallowing proinflammatory exposures from reaching the ovarian epithelium. This may account for the finding by Whittemore et al. (106), who reported no protective effect of hysterectomy in women who had a prior bilateral tubal ligation but found a reduction in risk for women with no prior tubal

ligation. Furthermore, Whittemore et al. showed that tubal ligation protected against the effect of talc. Women who used talc but had never had surgical sterilization were at 30% increased risk of cancer, whereas women who used talc but had a tubal ligation had a 50% reduction in risk. Thus, talc exposure may occur via ascension of particles from the lower to the upper part of the genital tract and tubal ligation severs this route of ovarian exposure. However, the risk reduction associated with tubal ligation or hysterectomy may be larger than would be expected, presuming that these procedures protect the ovarian epithelium from exposure to known inflammants, particularly because only a subset of women is exposed to talc or asbestos. The probable explanation for the fact that risk reduction for tubal ligation hysterectomy is larger than expected lies in the role of as yet unidentified environmental exposures. For example, sexually transmitted pathogens may act via inflammation to increase risk (see below). The inflammation hypothesis challenges investigators to search for other exposures that may gain access to the upper genital tract through the lower genital tract and initiate an inflammatory response.

PELVIC INFLAMMATORY DISEASE

PID is a condition consisting of inflammation of the endometrium, tubes, and ovaries as a result of sexually transmitted infections that ascend from the lower to the upper part of the genital tract. Two case–control studies (34,116) have linked PID with ovarian cancer risk. A third study (117), in which a very small proportion of women (and, therefore, total number of women) reported previous PID, did not. The latter study (117) is likely limited not only by power but also potentially by underreporting of prior PID. Shu et al. (34) first reported a substantial but statistically nonsignificant relationship (OR = 3.0; 95% CI = 0.3–30.2) among a handful of affected case patients and control subjects in Shanghai, China. Risch and Howe (116) subsequently demonstrated the relationship in a study involving 450 case patients with ovarian cancer and 565 control subjects residing in and around Toronto, Canada. They found an increased risk of ovarian cancer among women who had had an episode of PID (OR = 1.5; 95% CI = 1.1–2.1). The relationship between PID and ovarian cancer was most evident in women who had had PID at an early age, were nulliparous, and were infertile. Moreover, there was an increasing trend in risk with increasing number of PID episodes. Each episode of PID promotes a greater and greater inflammatory response, resulting in increasing damage to ovarian and tubal structures and a greater chance of tubal infertility (which, if occurring before the first birth, would manifest itself as nulliparity). Indeed, in the previously mentioned retrospective study of the cohort of infertile women (64), those with tubal infertility were at a threefold increased risk of ovarian cancer. The RR for tubal infertility was of the same order of magnitude as it was for ovulatory infertility, albeit involving a smaller number of individuals and not reaching statistical significance. PID produces infertility by causing inflammation of and damage to the fallopian tube wherein the ovum reaches the uterus, rather than by any effect on ovulation (see below). Thus, the finding that PID is associated with ovarian cancer, particularly when there has been resultant chronic inflammation and infertility, is consistent with an inflammatory origin for ovarian cancer.

ANTI-INFLAMMATORY MEDICATIONS

One way to evaluate the role of inflammation in ovarian cancer is to examine the effect of anti-inflammatory medications on risk. Cramer et al. *(52)* asked 563 case patients with ovarian cancer and 523 population-based control subjects about their lifetime history of anti-inflammatory medication use. The OR for ovarian cancer associated with at least 6 months of once-per-week aspirin use was 0.75 (95% CI = 0.52–1.10) and for ibuprofen use was 1.03 (95% CI = 0.64–1.64). Limitations of this study included the modest number of case patients exposed to long-term aspirin use and the smaller number exposed to ibuprofen, which resulted in broad CIs around ORs; the inclusion of women with modest use of nonsteroidal anti-inflammatory medications as exposed; and the lack of dose or duration data for aspirin or ibuprofen use. Previous studies showing a protective benefit of aspirin use for colon cancer have typically used a more restrictive definition of exposure, such as aspirin use at least two to three times per week, and have more clearly shown an effect for aspirin use than for other nonsteroidal medications, predominantly because only for aspirin have the number of exposed individuals been sufficient to provide stable estimates *(118)*. Indeed, in the only other published study examining the role of analgesics on ovarian cancer risk *(89)*, among 189 women with epithelial ovarian cancers, the adjusted RR for infrequent use was 0.78 (not statistically significant), whereas the adjusted RR for frequent use was 0.51 (*P* = .05). Thus, further investigation of the impact of anti-inflammatory medications on ovarian cancer is warranted.

BIOLOGIC RATIONALE FOR THE ROLE OF INFLAMMATION IN OVARIAN CANCER RISK

Ames et al. *(119)* argued that carcinogenesis in general may be mediated by oxidative damage to DNA. The general theory was based on the finding that mutations in several critical genes, such as the p53 tumor suppressor gene, can lead to tumors. Damage to the DNA constituting these genes may contribute to mutagenicity, to a degree that depends on the degree of damage, the effectiveness of endogenous repair mechanisms, and the rates of cell division. More rapidly dividing cells would be most prone to errors in DNA replication and repair *(120)*.

Inflammation, by its nature, produces toxic oxidants meant to kill pathogens. These oxidants cause direct damage to DNA, proteins, and lipids and may, therefore, play a direct role in carcinogenesis *(121)*. At the same time, chronic inflammation is associated with increased cell division. Rapid cell division gives rise to the potential for replication errors with resultant DNA repair; aberrant DNA repair, particularly at key regulatory sites (e.g., tumor suppressor DNA regions), may increase the risk for mutagenesis *(119)*. Finally, bioactive substances, such as cytokines, growth factors, and prostaglandins, that are synonymous with inflammation may play an important role in ovarian mutagenesis. Ovarian epithelial cells secrete cytokines, including interleukin 1, interleukin 6, and macrophage colony-stimulating factor, among others *(122)*. Auersperg et al. *(123)* pointed out that these same factors are also produced by ovarian cancer cells and suggested that the recruitment of normally secreted cytokines into disregulated autocrine loops may be important in neoplastic progression. Prostaglandins have multiple effects that favor tumorigenesis *(124)*. For example, prostaglandins are more common in ovarian malignant tumors than in normal cells *(125)*,

overexpression of prostaglandins increases the invasiveness of tumor cells, and inhibitors of cyclooxygenase activity (and therefore prostaglandin formation) protect against a variety of cancers in animals *(124)*. Epidemiologic studies have shown that long-term use of nonsteroidal anti-inflammatory medications generally reduces the risk of colon cancer in both men and women *(118,126,127)* and breast cancer in women *(128)*.

Ovulation may be mutagenic. The process of ovulation requires disruption of the ovarian epithelium *(129,130)*. Degenerative epithelial cells adjacent to the site of follicular rupture are shed from the ovarian surface, presumably through apoptosis (i.e., programmed cell death). The wound that ensues from cell loss and follicular extrusion is repaired by the proliferation of epithelial cells from the perimeter of the ruptured follicle. In the process, inclusion cysts are formed as surface epithelial cells become entrapped in the ovarian wound created during ovulation. There has been speculation that inclusion cysts are among the ovarian surface changes that represent a path of differentiation that is less plastic than the relatively pleuripotential normal ovarian epithelium and more likely to proceed to ovarian carcinogenesis *(130)*. This suggestion comes from two observations. First, women with ovarian cancer are more likely to have inclusion cysts in the contralateral ovary *(131)*; however, this finding was not confirmed in another study *(132)*. Second, in an unblinded study *(133)*, ovaries of women at high familial risk of developing ovarian cancer, compared with ovaries of normal women, were more likely to have multiple inclusion cysts as well as papillomatosis, deep invaginations, epithelial pseudostratification, and/or hyperactive stroma. Women with a genetic predisposition to ovarian cancer may thus have ovarian epithelium that is already committed to ovarian carcinogenesis, a feature of which is an excess of inclusion cysts.

There are also data from animal studies and limited human studies to support the hypothesis that ovulation may trigger cellular events that result in carcinogenesis. Hyperovulatory hens have a markedly increased likelihood of developing ovarian adenocarcinomas, as do rats with hyperproliferating ovarian epithelial cells *(134,135)*. In women, mutations of the p53 tumor suppressor gene were associated with an increased number of lifetime ovulations in a study by Schildkraut et al. *(120)*. Mutations of the p53 gene are the most common molecular alterations in ovarian cancer and are thought to result from spontaneous errors of DNA synthesis during cell proliferation *(136)*. Risch *(137)* questioned the validity of these results on the basis that case patients with p53 mutations were older, had poorer tumor differentiation, and had disease of distant rather than of local or regional stage at diagnosis, perhaps indicating that p53-positive tumors are diagnosed later in the neoplastic process. Schildkraut et al. *(138)* reanalyzed the data matching on age and then on stage and replicated the original findings. However, a more recent case–control study *(139)* was unable to confirm the association between lifetime ovulations and p53 mutations.

Mutagenicity induced by ovulation may be mediated by inflammation. Ovulation is associated with a marked inflammatory process at the level of ovulatory follicles *(140)*. Many inflammatory mediators, including vasoactive agents such as bradykinin and inflammatory and anti-inflammatory substances such as prostaglandins and leukotrienes, are locally elevated during ovulation. Epithelium in the neighborhood of inclusion cysts is brought in closer proximity to these substances. Follicle rupture probably involves tissue remodeling, with high cell turn-

over, that is also characteristic of inflammatory reactions. Thus, the process of ovulation is intimately related to inflammation. In particular, epithelium in and around the site of ovulation may replicate more actively, come into contact with cytokines and prostaglandins, and may be subject to oxidative stress, thereby enhancing the risk of mutagenesis.

PREDICTIONS FROM THE INFLAMMATION HYPOTHESIS AND SUGGESTIONS FOR FUTURE RESEARCH

Direct induction of inflammation as a result of endometriosis, talc and asbestos exposure, and PID, as well as ovulation itself, may act to promote ovarian tumorigenesis. There would be several ways to help demonstrate the veracity of this hypothesis. First, anti-inflammatory medications should reduce the occurrence of ovarian cancer. Aspirin use was associated with a reduction in ovarian cancer risk in one previous epidemiologic study; ibuprofen was not *(52)*. Further studies are needed to examine this association. Populations of women with substantial exposures to anti-inflammatory medications, such as those with connective tissue diseases, may be at lower than expected risk, as long as their disease does not inflame the ovarian epithelium. The only study, to our knowledge, that has assessed ovarian cancer risk in a population with connective tissue disease was a relatively retrospective cohort study of patients with rheumatoid arthritis. Cibere et al. *(141)* examined the observed versus expected rates of numerous cancers among a cohort of 862 Canadian patients with rheumatoid arthritis followed for a mean of 17.4 years. Only five patients developed ovarian cancer, for a standardized incidence ratio of 0.89, which was not statistically significant. Although the number of observed cases was somewhat lower than expected, the number of cases was far too limited for clear interpretation. Larger studies would be of great interest.

Experimentally induced inflammation of the epithelial ovarian surface should be studied to see whether such manipulation will result in epithelial inclusion cysts. Furthermore, demonstration of markers of mutagenicity within inclusion cysts should be sought to suggest movement along a pathway toward ovarian cancer. For example, known markers of mutagenesis, such as mutations in tumor suppressor genes, if they are more common in inflammation-induced inclusion cysts, would provide evidence supporting the role of inflammation in ovarian cancer pathogenesis. Animal experiments could also examine whether suppression of ovarian epithelial inflammation with anti-inflammatory medications would reduce the number of inclusion cysts and the rate of cancer-associated mutations. Antioxidants may also lower ovarian cancer risk, and evaluation of such an effect in both animals and humans would be helpful in testing the inflammation hypothesis.

Susceptibility to the effects of ovarian epithelial inflammation may be modulated by DNA excision and repair potential; i.e., individuals with more precise or active DNA repair capabilities may be relatively spared from the effects of local inflammation. The prevalence of such DNA polymorphisms within women with ovarian cancer and control subjects could be tested. All of these are testable hypotheses that could help in our understanding of the biologic mechanisms underlying ovarian cancer.

It is likely that hypotheses regarding ovulation, gonadotropins, and inflammation are not mutually exclusive but are instead interactive. The occurrence of inflammation during ovulation has been discussed. Steroid hormones may also mediate inflammation. Estrogens, according to the gonadotropin hypothesis, elevate ovarian cancer risk and they may also stimulate the immune response *(142)*. In particular, estrogens have been demonstrated *in vitro* to stimulate B-cell response and decrease suppressor T-cell reactivity, resulting in elevations in antibodies and autoantibodies. Moreover, oral contraceptives elevate the concentrations of local immunoglobulin G and immunoglobulin A in the female genital tract *(143)*. Elevated LH may also enhance oxidative stress. The principal bioassay for LH is the ovarian ascorbic acid depletion assay. Ascorbic acid is an antioxidant, and it is possible that LH depletes ascorbic acid by generating the production of free radicals *(144)*. This observation—that a gonadotropin and estrogen may stimulate inflammation and oxidation—provides a link between steroid hormone excess and the physiologic events involved in inflammation. Thus, it is not necessary to argue as to whether the data fit one hypothesis better than another, but rather it is necessary to develop a more comprehensive model of pathogenesis that may incorporate a role for steroid hormones, ovulation, and inflammation in ovarian cancer. Such a model would account for epidemiologic data suggesting associations between reproductive factors and ovarian cancer and also between PID, endometriosis, talc and asbestos exposure, tubal ligation, and hysterectomy and ovarian cancer.

SUMMARY

Neither incessant ovulation nor gonadotropin stimulation of ovarian estrogen provides a completely satisfactory explanation for the genesis of ovarian cancer. We have reviewed the data suggesting that an additional mechanism that may underlie ovarian cancer is inflammation, with concomitant rapid DNA turnover and defective repair, oxidative stress, and elevation of bioactive substances. Incessant ovulation, a process that has been linked to ovarian cancer risk, is associated with inflammation at the level of both the epithelium and the follicle. Other factors that cause local pelvic inflammation may also increase risk. Finally, tubal ligation and hysterectomy, which diminish the potential that ovarian epithelium will be exposed to initiators of inflammation, reduce risk. Further observational and experimental data will be needed to confirm the hypothesis that inflammation is a central biologic process in ovarian cancer risk.

REFERENCES

(1) Landis SH, Murray T, Bolden S, Wingo PA. Cancer statistics, 1998 [published erratum appears in CA Cancer J Clin 1998;48:192 and 1998;48:329]. CA Cancer J Clin 1998;48:6–29.

(2) Fathalla MF. Incessant ovulation—a factor in ovarian neoplasia? Lancet 1971;2:163.

(3) Russell SE, Hickey GI, Lowry WS, White P, Atkinson RJ. Allele loss from chromosome 17 in ovarian cancer. Oncogene 1990;5:1581–3.

(4) Eccles DM, Cranston G, Steele CM, Nakamura Y, Leonard RC. Allele losses on chromosome 17 in human epithelial ovarian carcinoma. Oncogene 1990;5:1599–601.

(5) Cramer DW, Welch WR. Determinants of ovarian cancer risk. II. Inferences regarding pathogenesis. J Natl Cancer Inst 1983;71:717–21.

(6) Risch HA, Marrett LD, Howe GR. Parity, contraception, infertility, and the risk of epithelial ovarian cancer. Am J Epidemiol 1994;140:585–97.

(7) Risch HA. Estrogen replacement therapy and risk of epithelial ovarian cancer. Gynecol Oncol 1996;63:254–7.

(8) Booth M, Beral V, Smith P. Risk factors for ovarian cancer: a case–control study. Br J Cancer 1989;60:592–598.

(9) McGowan L, Parent L, Lednar W, Norris HJ. The women at risk for developing ovarian cancer. Gynecol Oncol 1979;7:325–44.

(10) Gwinn ML, Lee NC, Rhodes PH, Layde PM, Rubin GL. Pregnancy, breast feeding, and oral contraceptives and the risk of epithelial ovarian cancer. J Clin Epidemiol 1990;43:559–68.

(11) Tzonou A, Day NE, Trichopoulos D, Walker A, Saliaraki M, Papapostolou M, et al. The epidemiology of ovarian cancer in Greece. A case–control study. Eur J Cancer Clin Oncol 1984;20:1045–52.

(12) Hildreth NG, Kelsey JL, LiVolsi VA, Fischer DB, Holford TR, Mostow ED, et al. An epidemiologic study of epithelial carcinoma of the ovary. Am J Epidemiol 1981;114:398–405.

(13) Newhouse ML, Pearson RM, Fullerton JM, Boesen EA, Shannon HS. A case control study of the ovary. Br J Prev Soc Med 1977;31:148–53.

(14) Wu ML, Whittemore AS, Paffenbarger RS Jr, Sarles DL, Kampert JB, Grosser S, et al. Personal and environmental characteristics related to epithelial ovarian cancer. I. Reproductive and menstrual events and oral contraceptive use. Am J Epidemiol 1988; 128:1216–27.

(15) Casagrande JT, Louie EW, Pike MC, Roy S, Ross RK, Henderson BE. "Incessant ovulation" and ovarian cancer. Lancet 1979;2:170–3.

(16) Rosenberg L, Shapiro S, Slone D, Kaufman DW, Helmrich SP, Miettinen OS, et al. Epithelial ovarian cancer and combination oral contraceptives. JAMA 1982;247:3210–2.

(17) Mori M, Harabuchi I, Miyake H, Casagrande JT, Henderson BE, Ross RK. Reproductive, genetic, and dietary risk factors for ovarian cancer. Am J Epidemiol 1988;128:771–7.

(18) Cramer DW, Hutchison GB, Welch WR, Scully RE, Ryan KJ. Determinants of ovarian cancer risk. I. Reproductive experiences and family history. J Natl Cancer Inst 1983;1:711–6.

(19) Kvale G, Heuch I, Nilssen S, Beral V. Reproductive factors and risk of ovarian cancer: a prospective study. Int J Cancer 1988;42:246–51.

(20) Joly DJ, Lilienfeld AM, Diamond EL, Bross ID. An epidemiologic study of the relationship of reproductive experience to cancer of the ovary. Am J Epidemiol 1974;99:190–209.

(21) Nasca PC, Greenwald P, Chorost S, Richart R, Caputo T. An epidemiologic case–control study of ovarian cancer and reproductive factors. Am J Epidemiol 1984;119:705–13.

(22) Franceschi S, La Vecchia C, Helmrich SP, Mangioni C, Tognoni G. Risk factors for epithelial ovarian cancer in Italy. Am J Epidemiol 1982;115:714–9.

(23) La Vecchia C, Decarli A, Franceschi S, Regallo M, Tognoni G. Age at first birth and the risk of epithelial ovarian cancer. J Natl Cancer Inst 1984;73:663–6.

(24) Risch HA, Weiss NS, Lyon JL, Daling JR, Liff JM. Events of reproductive life and the incidence of epithelial ovarian cancer. Am J Epidemiol 1983;117:128–39.

(25) Voigt LF, Harlow BL, Weiss NS. The influence of age at first birth and parity on ovarian cancer risk. Am J Epidemiol 1986;124:490–1.

(26) Mori M, Kiyosawa H, Miyake H. Case–control study of ovarian cancer in Japan. Cancer 1984;53:2746–52.

(27) Wynder EL, Dodo H, Barber HR. Epidemiology of cancer of the ovary. Cancer 1969;23:352–70.

(28) Hankinson SE, Colditz GA, Hunter DJ, Willett WC, Stampfer MJ, Rosner B, et al. A prospective study of reproductive factors and risk of epithelial ovarian cancer. Cancer 1995;76:284–90.

(29) Adami HO, Hsieh CC, Lambe M, Trichopoulos D, Leon D, Persson I, et al. Parity, age at first childbirth, and risk of ovarian cancer. Lancet 1944; 344:1250–4.

(30) Chen MT, Cook LS, Daling JR, Weiss NS. Incomplete pregnancies and risk of ovarian cancer (Washington, United States). Cancer Causes Control 1996;7:415–20.

(31) Whittemore AS, Harris R, Itnyre J. Characteristics relating to ovarian cancer risk: collaborative analysis of 12 US case–control studies. II. Invasive epithelial ovarian cancers in white women. Collaborative Ovarian Cancer Group. Am J Epidemiol 1992;136:1184–203.

(32) Purdie D, Green A, Bain C, Siskind V, Ward B, Hacker N, et al. Reproductive and other factors and risk of epithelial ovarian cancer: an Australian case–control study. Survey of Women's Health Study Group. Int J Cancer 1995;62:678–84.

(33) Hartge P, Schiffman MH, Hoover R, McGowan L, Lesher L, Norris HJ. A case–control study of epithelial ovarian cancer. Am J Obstet Gynecol 1989;161:10–6.

(34) Shu XO, Brinton LA, Gao YT, Yuan JM. Population-based case–control study of ovarian cancer in Shanghai. Cancer Res 1989;49:3670–4.

(35) Vessey M, Metcalfe A, Wells C, McPherson K, Westhoff C, Yeates D. Ovarian neoplasms, functional ovarian cysts, and oral contraceptives. Br Med J (Clin Res Ed) 1987;294:1518–20.

(36) Harlow BL, Weiss NS, Roth GJ, Chu J, Daling JR. Case–control study of borderline ovarian tumors: reproductive history and exposure to exogenous female hormones. Cancer Res 1988;48:5849–52.

(37) The Cancer and Steroid Hormone Study of the Centers for Disease Control and the National Institute of Child Health and Human Development. The reduction in risk of ovarian cancer associated with oral-contraceptive use. N Engl J Med 1987;316:650–5.

(38) The WHO Collaborative Study of Neoplasia and Steroid Contraceptives. Epithelial ovarian cancer and combined oral contraceptives. Int J Epidemiol 1989;18:538–45.

(39) Beral V, Hannaford P, Kay C. Oral contraceptive use and malignancies of the genital tract. Results from the Royal College of General Practitioners' Oral Contraceptive Study. Lancet 1988;2:1331–5.

(40) Willett WC, Bain C, Hennekens CH, Rosner B, Speizer FE. Oral contraceptives and risk of ovarian cancer. Cancer 1981;48:1684–7.

(41) Cramer DW, Hutchison GB, Welch WR, Scully RE, Knapp RC. Factors affecting the association of oral contraceptives and ovarian cancer. N Engl J Med 1982;307:1047–51.

(42) Weiss NS, Lyon JL, Liff JM, Vollmer WM, Daling JR. Incidence of ovarian cancer in relation to the use of oral contraceptives. Int J Cancer 1981;28:669–71.

(43) La Vecchia C, Franceschi S, Decarli A. Oral contraceptives and the risk of epithelial ovarian cancer. Br J Cancer 1984;50:31–4.

(44) Parazzini F, La Vecchia C, Negri E, Bocciolone L, Fedele L, Franceschi S. Oral contraceptive use and the risk of ovarian cancer: an Italian case–control study. Eur J Cancer 1991;27:594–8.

(45) Stanford JL. Oral contraceptives and neoplasia of the ovary. Contraception 1991;43:543–56.

(46) Whittemore AS, Harris R, Itnyre J. Characteristics relating to ovarian cancer risk: collaborative analysis of 12 US case–control studies. IV. The pathogenesis of epithelial ovarian cancer. Collaborative Ovarian Cancer Group. Am J Epidemiol 1992;136:1212–20.

(47) Liu J, Rebar RW, Yen SS. Neuroendocrine control of the postpartum period. Clin Perinatol 1983;10:723–36.

(48) Risch HA. Hormonal etiology of epithelial ovarian cancer, with a hypothesis concerning the role of androgens and progesterone. J Natl Cancer Inst 1998;90:1774–86.

(49) Glud E, Kjaer SK, Troisi R, Brinton LA. Fertility drugs and ovarian cancer. Epidemiol Rev 1998;20:237–57.

(50) Bristow RE, Karlan BY. Ovulation induction, infertility, and ovarian cancer risk. Fertil Steril 1996;66:499–507.

(51) Zeimet AG, Muller-Holzner E, Marth C, Daxenbichler G. Immunocytochemical versus biochemical receptor determination in normal and tumorous tissues of the female reproductive tract and the breast. J Steroid Biochem Mol Biol 1994;49:365–72.

(52) Cramer DW, Harlow BL, Titus-Ernstoff L, Bohlke K, Welch WR, Greenberg ER. Over-the-counter analgesics and risk of ovarian cancer. Lancet 1998;351:104–7.

(53) Cramer DW. Epidemiologic aspects of early menopause and ovarian cancer. Ann N Y Acad Sci 1990;592:363–75.

(54) Helzlsouer KJ, Alberg AJ, Gordon GB, Longcope C, Bush TL, Hoffman SC, et al. Serum gonadotropins and steroid hormones and the development of ovarian cancer. JAMA 1995;274:1926–30.

(55) Hoover R, Gray LA Sr, Fraumeni JF Jr. Stilboestrol (diethylstilbestrol) and the risk of ovarian cancer. Lancet 1977;2:533–4.

(56) Annegers JF, Strom H, Decker DG, Dockerty MB, O'Fallon WM. Ovarian cancer: incidence and case–control study. Cancer 1979;43:723–9.

(57) Hartge P, Hoover R, McGowan L, Lesher L, Norris HJ. Menopause and ovarian cancer. Am J Epidemiol 1988;127:990–8.

(58) Weiss NS, Lyon JL, Krishnamurthy S, Dietert SE, Liff JM, Daling JR. Noncontraceptive estrogen use and the occurrence of ovarian cancer. J Natl Cancer Inst 1982;68:95–8.

(59) Kaufman DW, Kelly JP, Welch WR, Rosenberg L, Stolley PD, Warshauer ME, et al. Noncontraceptive estrogen use and epithelial ovarian cancer. Am J Epidemiol 1989;130:1142–51.

(60) La Vecchia C, Liberati A, Franceschi S. Noncontraceptive estrogen use and the occurrence of ovarian cancer [letter]. J Natl Cancer Inst 1982;69: 1207.

(61) Hempling RE, Wong C, Piver MS, Natarajan N, Mettlin CJ. Hormone replacement therapy as a risk factor for epithelial ovarian cancer: results of a case–control study. Obstet Gynecol 1997;89:1012–6.

(62) Rodriguez C, Calle EE, Coates RJ, Miracle-McMahill HL, Thurn MJ, Heath CW Jr. Estrogen replacement therapy and fatal ovarian cancer. Am J Epidemiol 1995;141:828–35.

(63) Garg PP, Kerlikowske K, Subak L, Grady D. Hormone replacement therapy and the risk of epithelial ovarian carcinoma: a meta-analysis. Obstet Gynecol 1998;92:472–9.

(64) Rossing MA, Daling JR, Weiss NS, Moore DE, Self SG. Ovarian tumors in a cohort of infertile women. N Engl J Med 1994;331:771–6.

(65) Venn A, Watson L, Lumley J, Giles G, King C, Healy D. Breast and ovarian cancer incidence after infertility and *in vitro* fertilisation. Lancet 1995;346:995–1000.

(66) Bristow RE, Karlan BY. Ovulation induction, infertility, and ovarian cancer risk. Fertil Steril 1996;66:499–507.

(67) Mosgaard BJ, Lidegaard O, Kjaer SK, Schou G, Andersen AN. Infertility, fertility drugs, and invasive ovarian cancer: a case–control study. Fertil Steril 1997;67:1005–12.

(68) Modan B, Ron E, Lerner-Geva L, Blumstein T, Menczer J, Rabinovici J, et al. Cancer incidence in a cohort of infertile women. Am J Epidemiol 1998;147:1038–42.

(69) Shushan A, Paltiel O, Iscovich J, Elchalal U, Peretz T, Schenker JG. Human menopausal gonadotropin and the risk of epithelial ovarian cancer. Fertil Steril 1996;65:13–8.

(70) Harlow SD, Ephross SA. Epidemiology of menstruation and its relevance to women's health. Epidemiol Rev 1995;17:265–86.

(71) Graham J, Graham R. Ovarian cancer and asbestos. Environ Res 1967; 1:115–28.

(72) Keal EE. Asbestosis and abdominal neoplasma. Lancet 1960;2:1211–6.

(73) Acheson ED, Gardner MJ, Pippard EC, Grime LP. Mortality of two groups of women who manufactured gas masks from chrysotile and crocidolite asbestos: a 40-year follow-up. Br J Ind Med 1982;39:344–8.

(74) Newhouse ML, Berry G, Wagner JC, Turok ME. A study of the mortality of female asbestos workers. Br J Ind Med 1972;29:134–41.

(75) Newhouse ML, Berry G, Wagner JC. Mortality of factory workers in East London 1933–80. Br J Ind Med 1985;42:4–11.

(76) Heller DS, Gordon RE, Westhoff C, Gerber S. Asbestos exposure and ovarian fiber burden. Am J Ind Med 1996;29:435–9.

(77) Kelsey JL, Hildreth NG. Breast and gynecologic cancer epidemiology. Boca Raton (FL): CRC press; 1983.

(78) Neugut AI, Eisenberg D, Silverstein M, Pulkrabek P, Weinstein IB. Effects of asbestos on epithelioid cell lines. Environ Res 1978;17:256–65.

(79) Rice PA, Schachter J. Pathogenesis of pelvic inflammatory disease. What are the questions? JAMA 1991;266:2587–93.

(80) Harlow BL, Hartge PA. A review of perineal talc exposure and risk of ovarian cancer. Regul Toxicol Pharmacol 1995;21:254–60.

(81) Cook LS, Kamb ML, Weiss NS. Perineal powder exposure and the risk of ovarian cancer [published erratum appears in Am J Epidemiol 1998;148: 410]. Am J Epidemiol 1997;145:459–65.

(82) Cramer DW, Welch WR, Scully Re, Wojciechowski CA. Ovarian cancer and talc: a case–control study. Cancer 1982;50:372–6.

(83) Whittemore AS, Wu ML, Paffenbarger PS Jr, Sarles DL, Kampert JB, Grosser S, et al. Personal and environmental characteristics related to epithelial ovarian cancer. II. Exposures to talcum powder, tobacco, alcohol, and coffee. Am J Epidemiol 1988;128:1228–40.

(84) Harlow BL, Weiss NS. A case–control study of borderline ovarian tumors: the influence of perineal exposure to talc. Am J Epidemiol 1989; 130:390–4.

(85) Chen Y, Wu PC, Lang JH, Ge WJ, Hartge P, Brinton LA. Risk factors for epithelial ovarian cancer in Beijing, China. Int J Epidemiol 1992;21:23–9.

(86) Harlow BL, Cramer DW, Bell DA, Welch WR. Perineal exposure to talc and ovarian cancer risk. Obstet Gynecol 1992;80:19–26.

(87) Rosenblatt KA, Szklo M, Rosenshein NB. Mineral fiber exposure and the development of ovarian cancer. Gynecol Oncol 1992;45:20–5.

(88) Hartge P, Hoover R, Lesher LP, McGowan L. Talc and ovarian cancer [letter]. JAMA 1988;250:1844.

(89) Tzonou A, Polychronopoulou A, Hsieh CC, Rebelakos A, Karakatsani A, Trichopoulos D. Hair dyes, analgesics, tranquilizers and perineal talc application as risk factors for ovarian cancer. Int J Cancer 1993;55:408–10.

(90) Chang S, Risch HA. Perineal talc exposure and risk of ovarian carcinoma. Cancer 1997;79:2396–401.

(91) Heller DS, Westhoff C, Gordon RE, Katz N. The relationship between perineal cosmetic talc usage and ovarian talc particle burden. Am J Obstet Gynecol 1996;174:1507–10.

(92) Harlow BL, Hartge PA. A review of perineal talc exposure and risk of ovarian cancer. Regul Toxicol Pharmacol 1995;21:254–60.

(93) Heaps JM, Nieberg RK, Berek JS. Malignant neoplasms arising in endometriosis. Obstet Gynecol 1990;75:1023–8.

(94) Hitti IF, Glasberg SS, Lubicz S. Clear cell carcinoma arising in extraovarian endometriosis: report of three cases and review of the literature. Gynecol Oncol 1990;39:314–20.

(95) Brinton LA, Gridley G, Persson I, Baron J, Bergqvist A. Cancer risk after a hospital discharge diagnosis of endometriosis. Am J Obstet Gynecol 1997;176:572–9.

(96) Sainz de la Cuesta R, Eichhorn JH, Rice LW, Fuller AF Jr, Mikrui N, Goff BA. Histologic transformation of benign endometriosis to early epithelial ovarian cancer. Gynecol Oncol 1996;60:238–44.

(97) Vercellini P, Parazzini F, Bolis G, Carinelli S, Dindelli M, Vendola N, et al. Endometriosis and ovarian cancer. Am J Obstet Gynecol 1993;169: 181–2.

(98) LaGrenade A, Silverberg SG. Ovarian tumors associated with atypical endometriosis. Hum Pathol 1988;19:1080–4.

(99) Moll UM, Chumas JC, Chalas E, Mann WJ. Ovarian carcinoma arising in atypical endometriosis. Obstet Gynecol 1990;75:537–9.

(100) Jimbo H, Yoshikawa H, Onda T, Yasugi T, Sakamoto A, Taketani Y. Prevalence of ovarian endometriosis in epithelial ovarian cancer. Int J Gynecol Obstet 1997;59:245–50.

(101) DePriest PD, Banks ER, Powell DE, van Nagell JR Jr, Gallion HH, Puls SE, et al. Endometrioid carcinoma of the ovary and endometriosis: the association in postmenopausal women. Gynecol Oncol 1992;47:71–5.

(102) Sampson JA. Endometrial carcinoma of the ovary arising in endometrial tissue in that organ. Arch Surg 1925;10:1–72.

(103) Friedlander ML. Prognostic factors in ovarian cancer. Semin Oncol 1998; 25:305–14.

(104) Czernobilsky B, Morris WJ. A histologic study of ovarian endometriosis with emphasis on hyperplastic and atypical changes. Obstet Gynecol 1979;53:311–23.

(105) Weiss NS, Harlow BL. Why does hysterectomy without bilateral oophorectomy influence the subsequent incidence of ovarian cancer? Am J Epidemiol 1986;124:856–8.

(106) Whittemore AS, Wu ML, Paffenbarger RS Jr, Sarles DL, Kampert JB, Grosser S, et al. Epithelial ovarian cancer and the ability to conceive. Cancer Res 1989;49:4047–52.

(107) Irwin KL, Weiss NS, Lee NC, Peterson HB. Tubal sterilization, hysterectomy, and the subsequent occurrence of epithelial ovarian cancer. Am J Epidemiol 1991;134:362–9.

(108) Cramer DW, Xu H. Epidemiologic evidence for uterine growth factors in the pathogenesis of ovarian cancer. Ann Epidemiol 1995;5:310–4.

(109) Hankinson SE, Hunter DJ, Colditz GA, Willett WC, Stampfer MJ, Rosner B, et al. Tubal ligation, hysterectomy, and risk of ovarian cancer. A prospective study. JAMA 1993;270:2813–8.

(110) Miracle-McMahill HL, Calle EE, Kosinski AS, Rodriguez C, Wingo PA, Thun MJ, et al. Tubal ligation and fatal ovarian cancer in a large prospective cohort study. Am J Epidemiol 1997;145:349–57.

(111) Loft A, Lidegaard O, Tabor A. Incidence of ovarian cancer after hysterectomy: a nationwide controlled follow up. Br J Obstet Gynaecol 1997; 104:1296–301.

(112) Kreiger N, Sloan M, Cotterchio M, Parsons P. Surgical procedures associated with risk of ovarian cancer. Int J Epidemiol 1997;26:710–5.

(113) Cornelison TL, Natarajan N, Piver MS, Mettlin CJ. Tubal ligation and the risk of ovarian carcinoma. Cancer Detect Prev 1997;21:1–6.

(114) Green A, Purdie D, Bain C, Siskind V, Russell P, Quinn M, et al. Tubal sterilisation, hysterectomy and decreased risk of ovarian cancer. Survey of Women's Health Study Group. Int J Cancer 1997;71:948–51.

(115) Rosenblatt KA, Thomas DB. Reduced risk of ovarian cancer in women with a tubal ligation or hysterectomy. The World Health Organization

Collaborative Study of Neoplasia and Steroid Contraceptives. Cancer Epidemiol Biomarkers Prev 1996;5:933–5.

(116) Risch HA, Howe GR. Pelvic inflammatory disease and the risk of epithelial ovarian cancer. Cancer Epidemiol Biomarkers Prev 1995;4: 447–51.

(117) Parazzini F, La Vecchia C, Negri E, Moroni S, dal Pino D, Fedele L. Pelvic inflammatory disease and risk of ovarian cancer. Cancer Epidemiol Biomarkers Prev 1996;5:667–9.

(118) Giovannucci E, Rimm EB, Stampfer MJ, Colditz GA, Ascherio A, Willett WC. Aspirin use and the risk for colorectal cancer and adenoma in male health professionals. Ann Intern Med 1994;121:241–6.

(119) Ames BN, Gold LS, Willett WC. The causes and prevention of cancer. Proc Natl Acad Sci U S A 1995;92:5258–65.

(120) Schildkraut JM, Bastos E, Berchuck A. Relationship between lifetime ovulatory cycles and overexpression of mutant p53 in epithelial ovarian cancer. J Natl Cancer Inst 1997;89:932–8.

(121) Dreher D, Junod AF. Role of oxygen free radicals in cancer development. Eur J Cancer 1996;32A:30–8.

(122) Ziltener HJ, Maines-Bandiera S, Schrader JW, Auersperg N. Secretion of bioactive interleukin-1, interleukin-6, and colony-stimulating factors by human ovarian surface epithelium. Biol Reprod 1993;49:635–41.

(123) Auersperg N, Edelson MI, Mok SC, Johnson SW, Hamilton TC. The biology of ovarian cancer. Semin Oncol 1998;25:281–304.

(124) Subbaramaiah K, Zakim D, Weksler BB, Dannenberg AJ. Inhibition of cyclooxygenase: a novel approach to cancer prevention. Proc Soc Exp Biol Med 1997;216:201–10.

(125) Aitokallio-Tallberg AM, Viinikka LU, Ylikorkala RO. Increased synthesis of prostacyclin and thromboxane in human ovarian malignancy. Cancer Res 1988;48:2396–8.

(126) Giovannucci E, Egan KM, Hunter DJ, Stampfer MJ, Colditz GA, Willett WC, et al. Aspirin and the risk of colorectal cancer in women. N Engl J Med 1995;333:609–14.

(127) Giardiello FM, Hamilton SR, Krush AJ, Piantadosi S, Hylind LM, Celano P, et al. Treatment of colonic and rectal adenomas with sulindac in familial adenomatous polyposis. N Engl J Med 1993;328:1313–6.

(128) Harris RE, Namboodiri KK, Farrar WB. Nonsteroidal antiinflammatory drugs and breast cancer. Epidemiolgy 1996;7:203–5.

(129) Murdoch WJ. Ovarian surface epithelium, ovulation and carcinogenesis. Biol Rev Camb Philos Soc 1996;71:529–43.

(130) Auersperg N, Maines-Bandiera SL, Dyck HG. Ovarian carcinogenesis and the biology of ovarian surface epithelium. J Cell Physiol 1997;173: 261–5.

(131) Mittal KR, Zeleniuch-Jacquotte A, Cooper JL, Demopoulos RI. Contralateral ovary in unilateral ovarian carcinoma: a search for preneoplastic lesions. Int J Gynecol Pathol 1993;12:59–63.

(132) Westhoff C, Murphy P, Heller D, Halim A. Is ovarian cancer associated with an increased frequency of germinal inclusion cysts? Am J Epidemiol 1993;138:90–3.

(133) Salazar H, Godwin AK, Daly MB, Laub PB, Hogan WM, Rosenblum N, et al. Microscopic benign and invasive malignant neoplasms and a cancer-prone phenotype in prophylactic oophorectomies. J Natl Cancer Inst 1996; 88:1810–20.

(134) Wilson JE. Adenocarcinomata in hens kept in a constant environment. Poultry Sci 1958;37:1253.

(135) Godwin AK, Testa JR, Handel LM, Liu Z, Vanderveer LA, Tracey PA, et al. Spontaneous transformation of rat ovarian surface epithelial cells: association with cytogenetic changes and implications of repeated ovulation in the etiology of ovarian cancer. J Natl Cancer Inst 1992;84:592–601.

(136) Jones PA, Buckley JD, Henderson BE, Ross RK, Pike MC. From gene to carcinogen: a rapidly evolving field in molecular epidemiology. Cancer Res 1991;51:3617–20.

(137) Risch HA. Relationship between lifetime ovulatory cycles and overexpression of mutant p53 in epithelial ovarian cancer [letter]. J Natl Cancer Inst 1997;89:1726–7.

(138) Schildkraut JM, Bastos E, Halabi S, Berchuck A. Relationship between lifetime ovulatory cycles and overexpression of mutant p53 in epithelial ovarian cancer. J Natl Cancer Inst 1997;89:932–8.

(139) Webb PM, Green A, Cummings MC, Purdie DM, Walsh MD, Chenevix-Trench G. Relationship between number of ovulatory cycles and accumulation of mutant p53 in epithelial ovarian cancer. J Natl Cancer Inst 1998;90:1729–34.

(140) Espey LL. Current status of the hypothesis that mammalian ovulation is comparable to an inflammatory reaction. Biol Reprod 1994;50:233–8.

(141) Cibere J, Sibley J, Haga M. Rheumatoid arthritis and the risk of malignancy. Arthritis Rheum 1997;40:1580–6.

(142) Manzi S, Ramsey-Goldman R. Autoimmune diseases. In: Ness RB, Kuller LH, editors. Health and disease among women. Biological and environmental influences. New York (NY): Oxford University Press; 1998. p. 342–72.

(143) Crowley-Nowick PA. Basic principles of immunology and genital tract immunity. In: Ness RB, Kuller LH, editors. Health and disease among women. Biological and environmental influences. New York (NY): Oxford University Press; 1998. p. 273–96.

(144) Kolodecik TR, Aten RF, Behrman HR. Ascorbic acid-dependent cytoprotection of ovarian cells by leukocyte and nonleukocyte peroxidases. Biochem Pharmacol 1998;9:1497–503.

NOTE

Exhibit 78

Sep.30. 2004 11:00AM   KENNECOTT ENERGY CO   No.3679   P. 1

Luzenac AMERICA

DENVER TECHNICAL CENTER
345 Inverness Drive South • Centennial, CO 80112 • USA

FACSIMILE

DATE: September 30, 2004

TO: **Bill Ashton**

cc:

FROM: Richard J. Zazenski
      Director Product Safety
PHONE: 303-643-0404
e-MAIL: rzazenski@luzenac.com
FAX: 303-643-0446

Number of Pages: 13 Pages
(including cover sheet)

Bill – I came across this paper this morning published in the April, 2004 journal "Human Reproduction", an official journal of the European Society for Human Reproduction and Embryology. It offers some compelling evidence in support of the "migration" hypothesis. Combine this "evidence" with the theory that talc deposition on the ovarian epithelium initiates epithelium inflammation -- which leads to epithelium carcinogenesis -- and you have a potential formula for NTP classifying talc as a causative agent in ovarian cancer.

Please note that the tables and figures cited in the paper are "pasted" after the References at the end of the paper.

| Epithelial inflammation initiation |
| Persistently high levels of steroid hormones, gonadotrophins |
| Ovulation |
| INFLAMMATION - DNA damage and repair • Oxidative stress • Elevated cytokines and prostaglandins |
| Hysterectomy and tubal ligation |
| OVARIAN CARCINOGENESIS |
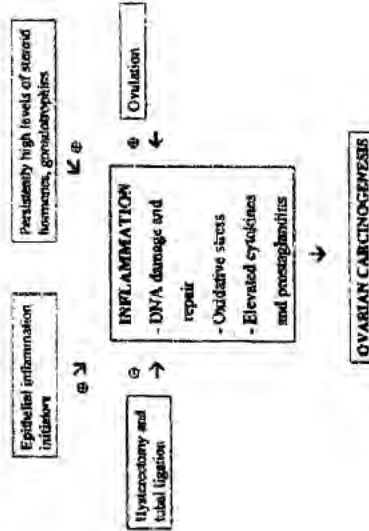
If any pages are unclear, please contact us.

Retrograde migration of glove powder in the human female genital tract

A.C.F. Sjösten[1], H. Ellis[2] and G.A.B. Edelstam[1]

[1]Karolinska Institutet, Department of Obstetrics & Gynaecology at Stockholm Söder Hospital, s-118 83 Stockholm, Sweden and [2]Department of Anatomy, Guy's, King's and St Thomas' School of Biomedical Sciences, London Bridge, London SE1 9RT, UK [3]To whom correspondence should be addressed. e-mail: anette.sjostam@sos.sll.se

▲ Abstract

BACKGROUND: This study in humans was undertaken to evaluate earlier results from animal research showing a retrograde migration of glove powder from the vagina into the intra-abdominal cavity. METHODS: One study group was gynaecologically examined with powdered gloves the day before an abdominal hysterectomy and another group 4 days pre-operatively. There were two control groups similarly examined with powder-free gloves. Cell smears were taken from the peritoneal fluid and during the operation further smears were taken from the Fallopian tubes, uterine cavity and cervical canal. RESULTS: Statistically significant differences were found for large starch particles at all locations between the study and control groups examined 1 day pre-operatively. Considering small starch particles, there were significant differences in cervix ($P <$ 0.001), uterus ($P < 0.01$) and the Fallopian tubes ($P < 0.01$). The combined results also show significant differences between both large and small starch particles in cervix, uterus and the Fallopian tubes. There were also differences between the study and control groups examined 4 days pre-operatively, but these were not statistically significant except for small and large starch particles in uterus ($P < 0.01$, $P < 0.05$) and cervix ($P < 0.05$, $P <$ 0.05). CONCLUSIONS: This study has pointed out a retrograde migration of starch also in humans after a gynaecological examination with powdered gloves. Consequently, powder or any other potentially harmful substance that can migrate from the vagina should be avoided.

Key words: female/gloves/retrograde migration/starch particles/vaginal examination

▲ Introduction

Earlier case reports suggest that intra-abdominal granulomas or adhesions due to starch particles were caused by starch powder used on gloves during vaginal examination. An initial indication of retrograde flow through the Fallopian tubes was the finding of intraperitoneal starch granulomas (Paine and Smith, 1957∗). Later the first case of starch peritonitis in a patient without previous surgery was reported (Saxen et al., 1963∗). A recent investigation detected talcum particles on the ovaries in women who had used perineal talc applications (Heller et al., 1996∗). In contrast, tubal ligation prevents the

Pltf_JNJ_00003742

Sep.30. 2004 11:01AM   KENNECOTT ENERGY CO   No.3879   P. 3

access of mediators that reach the peritoneal cavity through the Fallopian tubes (Ylikorkala, 2001◆).

Powder-free gloves have been available for 20 years, but starch-powdered gloves are still available and in use (Sjösten et al., 1999◆).

It is well documented that starch-powdered gloves are not appropriate for abdominal surgery (Ellis, 1990◆; Holmdahl et al., 1994◆), and intraperitoneally, starch particles can initiate inflammatory reaction and the formation of adhesions (Edelstam et al., 1992◆; diZerega, 1994◆), although the mechanism by which starch increases the propensity of tissues to form adhesions is not known. Reduced peritoneal fibrinolysis and activation of leukocytes by particulate starch granules have been suggested as possible mechanisms. Activated leukocytes, particularly macrophages, produce supernormal amounts of oxygen-free radicals, prostaglandin E_2, thromboxane B_2 and various cytokines (Osman and Jansen, 1999◆). Starch particles also increase the eicosanoid production which may contribute to the inflammatory or immune reactions and development of adhesions (Chegini and Rong, 1999◆). If already injured mesothelial surface of the peritoneum is exposed to starch, more dense adhesions are created compared to the effect of peritoneal trauma or starch separately. Application of glove powder on minimally or severely traumatized peritoneum facilitates tumour cell adhesion and growth alone (van den Tol et al., 2001◆). Histological re-evaluation after tubal reconstructive surgery due to peritubal or peri-ovarian adhesions has shown residual starch from powdered gloves (Yaffe et al., 1980◆).

A causal connection has been shown between operative tissue damage, intra-abdominal ischaemia, infections, reactions to foreign materials such as sutures, particles of gauze, glove dusting powder and post-operative adhesions (Myllärniemi, 1967◆; Holmdahl et al., 1996◆). One of the proven causes of post-operative intestinal adhesions is microscopic foreign bodies which are present in up to 93% of adhesions (Duron et al., 1997◆). After open abdominal or pelvic surgery, a third of the patients are readmitted at least twice during the subsequent 10 years for a disorder directly or possibly related to adhesions (Ellis et al., 1999◆).

Our previous investigation in a rabbit model indicated a retrograde migration of glove powder from the vagina into the intra-abdominal cavity (Edelstam et al., 1997◆). The amount that reaches the peritoneum is sufficient to significantly increase formation of post-operative adhesions after a standardized trauma (Sjösten et al., 2000◆).

Therefore, this subsequent study in humans was done to investigate whether starch particles from powdered gloves also in humans might gain access to the abdominal cavity through the vagina after a gynaecological examination with powdered gloves.

JNJ 000000706

Pltf_JNJ_00003742

A considerable number of gynaecologists wears starch-powdered gloves (Sjösten et al., 1999•), despite evidence of starch-induced complications. The starch particles can migrate not only from the vagina into the cervical canal and the uterine cavity but also through the Fallopian tubes into the peritoneal fluid. Women exposed to intra-abdominal surgical trauma 1–4 days after a gynaecological examination with powdered gloves may be at increased risk of intra-abdominal adhesions. But even without a surgical procedure there is a risk of intra-abdominal or peri-tubal adhesions due to the examination with powdered gloves (Osser et al., 1989•). Ongoing subclinical PID can cause infective tissue damage. An extensive study by Myllärniemi (1967•) showed that talc, starch powder and lint in the abdominal cavity tended to accumulate in the traumatized areas of the peritoneum so that the foreign material contaminating the peritoneal tissues could act together with other traumatizing conditions, possibly preventing the resorption of fibrinous adhesions. This corresponds to our previous finding in the rabbit model that starch particles deposited in the vagina can migrate in a retrograde direction from the vagina into the abdominal cavity and, combined with an intra-abdominal trauma, generate dense adhesions (Sjösten et al., 2000•). Since there are indications towards retrograde migration of powder, it must not be used regardless of cyclic variations or sexual activity.

In conclusion, our results show that starch particles can migrate from the vagina into the cervical canal, the uterine cavity and through the Fallopian tubes up to 4 days after a gynaecological examination with powdered gloves. Glove powder contributes to adverse intra-abdominal reactions, which include adhesion formation and adhesion-related complications such as chronic pelvic pain and bowel obstruction. Tubal and pelvic adhesions are a major cause of female infertility. Since evidence suggests that a retrograde migration could be a general mechanism, our recommendation is that we should be critical of harmful substances, e.g. glove powder, that could migrate from the vagina to abdominal cavity.

▲ Acknowledgements

▲ References

Chegini N and Kong H (1999) Postoperative exposure to glove powders modulates production of peritoneal eicosanoids during peritoneal wound healing. *Eur J Surg* 165,698–704.

di Zerega GS (1994) Contemporary adhesion prevention. *Fertil Steril* 61,219–235.

Duron JJ, Ellian N and Olivier O (1997) Post-operative peritoneal adhesions and foreign bodies. *Eur J Surg* 579(Suppl),15–16.

Sep.30. 2004 11:09AM   KENNECOTT ENERGY CO   No.3879   P. 5

Statistics

Non-parametric Mann–Whitney U-tests and Fisher's exact test were used and values are given as SEM for the group. Differences were considered significant at the $P < 0.001$, $P < 0.01$ and $P < 0.05$ levels. All statistical tests were computerized and carried out with statistics programs (Statistica™, Statsoft, USA).

▲ Results

Group I: examined 1 day pre-operatively with (i) powdered gloves (n = 17) and (ii) powder-free gloves (n = 15)

Starch particles were found in the cell smears with more particles found on the slides from the patients examined with powdered gloves. The differences were significant at all locations in the genital tract for small particles (cervix $P < 0.001$, uterus and Fallopian tubes $P < 0.01$) and large particles (cervix and uterus $P < 0.01$ and Fallopian tubes $P < 0.05$) but only for large particles in the peritoneal fluid ($P < 0.05$). However, in two patients examined with powdered gloves, no particles were found. On the contrary, in three patients examined with powder-free gloves, a few particles were found (Table 1 and Figure 1).
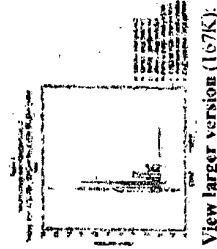
Figure 1. Median and range value for the retrograde transportation of small and large starch particles respectively, in different locations 1 day after a gynaecological examination with or without powdered gloves. The negative range value in the starch group for cervix, uterus and peritoneal fluid are due to contamination with airborne starch particles.

View larger version (167K):

Group II: examined 4 days pre-operatively with (i) powdered gloves (n = 12) and (ii) powder-free gloves (n = 14)

There were significantly more small starch particles as well as large particles (cervix and uterus $P < 0.05$) after examination with powdered gloves. The differences were the same for small particles but less significant for large particles (uterus $P < 0.05$). The differences were non-significant in the Fallopian tubes and the peritoneal fluid (Table II and Figure 2).

Pltf_JNJ_00003742

Materials and methods

Patients

The participants in the study were divided into four different groups. Informed consent was obtained from all participants. All had a routine gynaecological examination before an elective laparotomy for total or subtotal hysterectomy due to fibroids or menometrorrhagia. Group I: examined 1 day pre-operatively with (i) powdered gloves (Gammex® Ansell GmbH, Germany; $n = 17$, mean age 51 years) or (ii) powder-free gloves (Biogel® Regent Medical, SLL) ($n = 15$, mean age 51 years). Group II: examined 4 days pre-operatively with (i) powdered gloves ($n = 12$, mean age 53 years) or (ii) powder-free gloves ($n = 14$, mean age 52 years). Patients with cancer of the uterus were excluded as well as women with ongoing menstrual bleeding. The pre-menopausal women were examined regardless of the follicular or luteal phase of the menstrual cycle. A third of all women in the study were post-menopausal. Any medication that might have influenced the tubal patency had not been taken except in the case of three patients who had an asthmatic disease and needed to take terbutaline occasionally. The medication was not taken during the investigations. There were no other significant differences for patient characteristics. Sexual activity, cyclic changes or hormonal effect where not considered in this study.

Surgical procedure

An abdominal subtotal or total hysterectomy was undertaken with the operating team and the nurse who set up the instrument tray wearing powder-free gloves. Immediately the abdominal cavity was opened, peritoneal fluid was collected and cell smears were then taken from the peritoneal fluid. From the fimbriae of the Fallopian tubes, additional cell smears were taken per-operatively and when the uterus had been removed, i.e. post-operatively from the uterine cavity and the cervical canal. For making the smears sterile, forceps or pears were used. Smears from the fimbriae of the Fallopian tubes were omitted if they were not removed during the hysterectomy.

Cell smears

The cell smears were quantitatively standardized on ~1 cm^2 of one-half of a glass slide with the other blank side serving as control for contamination with air-borne starch particles. All the slides were stained with May-Grünewald Giemsa by a biochemical assistant wearing powder-free gloves in a laboratory where only powder-free gloves were used. The slides were coded and analysed by two independent investigators with a Zeiss 4/76 microscope using polarized light at magnification X250. The starch particles were counted in a standardized procedure for all slides. The numbers on the blank side (i.e. contamination) were subtracted from that in the smears so that the number of starch particles on each slide represent the net number without contaminating particles. Since there are differences in the size of starch particles they where divided into two sizes: (i) smaller than a leukocyte and (ii) larger than a leukocyte. Leukocytes for comparison in size were always present in the smears. The study was approved by the local ethics committee.
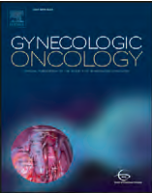
Pltf_JNJ_00003742

Exhibit 79

Review Article

Updates of the role of oxidative stress in the pathogenesis of ovarian cancer

CrossMark

Ghassan M. Saed [a,*], Michael P. Diamond [b], Nicole M. Fletcher [a]

[a] The Department of Obstetrics and Gynecology, Wayne State University School of Medicine, Detroit, MI, United States
[b] The Department of Obstetrics and Gynecology, Augusta University, Augusta, GA, United States

HIGHLIGHTS

- Oxidative stress plays an essential role in the pathogenesis of ovarian cancer.
- Modulating the redox balance may have therapeutic value.
- Chemoresistant ovarian cancer cells have an even further elevated oxidative stress.
- Chemotherapy induced mutations in redox enzymes may contribute to chemoresistance.

ARTICLE INFO

ABSTRACT

Clinical and epidemiological investigations have provided evidence supporting the role of reactive oxygen species (ROS) and reactive nitrogen species (RNS), collectively known as oxidative stress, in the etiology of cancer. Exogenous factors such as chronic inflammation, infection and hypoxia are major sources of cellular oxidative stress. Specifically, oxidative stress plays an important role in the pathogenesis, neoangiogenesis, and dissemination of local or distant ovarian cancer, as it is known to induce phenotypic modifications of tumor cells by cross talk between tumor cells and the surrounding stroma. Subsequently, the biological significance of the relationship between oxidative stress markers and various stages of epithelial ovarian cancer highlights potential therapeutic interventions as well as provides urgently needed early detection biomarkers. In the light of our scientific research and the most recent experimental and clinical observations, this review provides the reader with up to date most relevant findings on the role of oxidative stress in the pathogenesis of ovarian cancer and the possible therapeutic implications.

© 2017 Elsevier Inc. All rights reserved.

Contents

* Corresponding author at: Wayne State University, 275 E Hancock Street, Detroit, MI 48201, United States.
  E-mail address: gsaed@med.wayne.edu (G.M. Saed).

1. Ovarian cancer

Ovarian cancer is the fifth leading cause of cancer death; the leading cause of death from gynecologic malignancies, and the second most commonly diagnosed gynecologic malignancy; yet the underlying path ophysiology continues to be delineated [1]. The majority of advanced stage tumors are of epithelial cell origin and can arise from serous, mu cinous, or endometrioid cells on the surface epithelium of the ovary or the fallopian tube [1]. Surgical cytoreduction followed by platinum/ taxane chemotherapy results in complete clinical response in 50 80% of patients with stage III and IV disease, but most will relapse within 18 months with chemoresistant disease [1]. Mortality rates for this type of malignancy are high because of a lack of an early stage screening method, as well as the development of drug resistance [1].

Many cases of ovarian cancer continue to be described as de novo al though several theories regarding its origination have been proposed. Some of these theories include 1) the incessant ovulation hypothesis, where ovarian surface epithelial cells are injured due to repeated ovula tion leading to eventual transformation and malignancy, 2) the go nadotropin hypothesis describes overstimulation of ovarian surface epithelium through hormone receptors leading to malignant trans formation, and 3) the cell of origin for most epithelial ovarian cancer is not originating in the ovary but rather coming from the fallopian tube and spreading to the ovary, and beyond [1 3]. Thus, the exact origin(s) and pathogenesis of ovarian cancer still remains under debate.

Recently, a revised model of epithelial ovarian carcinogenesis has been proposed that distinguishes more clearly between type I and type II tumors based on both molecular genetic findings and histopath ologic studies [3]. Kurman and Shih describe a dualistic model of ovarian carcinogenesis where type I tumors develop from benign extraovarian precursor lesions that implant on the ovary are classified into three groups described as; endometriosis related tumors (endometrioid, clear cell, and seromucinous), low grade serous carcinomas, and then mucinous carcinomas and malignant Brenner tumors [3]. On the other hand, type II tumors develop from intraepithelial carcinomas in the fallopian tube, and involve both the ovary and extraovarian sites and are classified as high grade serous carcinomas that can be further subdivided into morphologic and molecular subtypes [3].

The overwhelming majority of ovarian cancers are derived from ovarian surface epithelium. Metastasis is achieved through detachment of single cells or clusters of cells from the primary tumor followed by implantation on peritoneal mesothelial lining [4]. Unlike many other type of cancer, ovarian carcinomas rarely metastasize outside of the peritoneal cavity [5]. Additionally, the presence of spheroids in ascites is a contributing factor to not only metastasis but also to chemoresistance. Spheroid cells are also known as ovarian cancer stem cells that have numerous characteristics of cancer stem cells in cluding self renewal, the ability to produce differentiated progeny, in creased expression of genes associated with cancer stem cells, higher invasiveness, migration potential, altered metabolism, and enhanced chemoresistance [4,6].

Ovarian cancer has also been characterized to manifest loss of func tion of the p53 gene due to mutations as well as other oncogenic path ways including retinoblastoma protein, the phosphatidylinositol 3 kinase (PI3K)/rat sarcoma viral oncogene pathways, and Notch signal ing [1]. Moreover, ovarian cancer is associated with germline mutations in the BRCA1 or BRCA2 genes, affecting only 20 40% of patients, suggest ing the possibility of the presence of unknown mutations in other genes [1]. Additional genetic variations, many of which have been identified in recent genome wide association studies, have been hypothesized to act as low to moderate penetrant alleles, which contribute to ovarian can cer risk, as well as other diseases [7]. Several studies have been done to identify differentially expressed genes in ovarian carcinoma for diag nosis of early stage ovarian cancer as well as the use of such markers as targets for improved therapy and treatment, although to date these have not yielded reproducible prognostic indicators for identification and clinical outcomes [1,8 10].

2. Oxidative stress

The imbalance between production and elimination of free radicals and reactive metabolites leads to a state of oxidative stress and subse quent damage of important biomolecules and cells, with potential im pact on the whole organism [11]. Reactive oxygen species (ROS) are oxygen derived small molecules, including oxygen radicals, such as su peroxide ($O_2\bullet^-$), hydroxyl (HO•), peroxyl (RO$_2$•), and alkoxyl (RO•), as well as various non radicals that can be converted to radicals or serve as oxidizing agents and include hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), ozone (O_3), and singlet oxygen ($^1O_2$) [11, 12]. Reactive nitrogen species (RNS) are nitrogen containing oxidants and are formed from nitric oxide (NO) that is generated from the mito chondrial respiratory chain under hypoxic conditions [11]. The persis tent generation of cellular ROS and RNS is a consequence of many factors including exposure to carcinogens, infection, inflammation, en vironmental toxicants, nutrients, and mitochondrial respiration [11 14]. Various enzyme systems produce ROS and RNS including cyto chrome P450, lipoxygenase, cyclooxygenase, nicotinamide adenine di nucleotide phosphate (NAD(P)H) oxidase complex, xanthine oxidase (XO), and peroxisomes [11,13,15] (Fig. 1).

Various enzyme systems that neutralize toxic ROS and RNS are vital in maintaining the redox balance, and are summarized in Fig. 1. Super oxide dismutase (SOD) catalyzes the conversion of $O_2\bullet^-$ to H_2O_2, which then can be converted to water by catalase (CAT) or glutathione perox idase (GPX) coupled with glutathione reductase (GSR) [12] (Fig. 1). Other important scavengers include thioredoxin coupled with thioredoxin reductase, and glutaredoxin, which utilizes glutathione (GSH) as a substrate. Additionally, glutathione S transferase (GST) is in volved in detoxification of varieties of environmental carcinogens and xenobiotics by catalyzing their conjugation to GSH, and subsequent re moval from the cell [12] (Fig. 1). Glutathione plays a central role in maintaining redox homeostasis, and the GSH to oxidized GSH (GSH/ GSSG) ratio provides an estimate of cellular redox buffering capacity [16,17]. Moreover, evidence suggests that increased oxidative stress mediated by the GSH/GSSG complex results in enhanced activity of the GS X MRP1 efflux pump [17]. This pump is known to decrease the intracellular effective chemotherapeutic drug concentration; therefore it is considered one of the mechanisms of multiple drug resistance [16, 17].

3. Oxidative stress and cancer

Oxidative stress has been reported to affect all phases of the onco genic process including initiation, promotion, and progression [11,12]. Oxidative stress is known to activate several transcription factors in cluding nuclear factor (NF) κB, activator protein (AP) 1, p53, hypoxia inducible factor (HIF) 1α, peroxisome proliferator activated receptor (PPAR) γ, β catenin/Wnt, and Nuclear factor erythroid 2 related factor 2 (Nrf2), which modulate the expression of numerous genes involved in immune and inflammatory responses, tissue remodeling and fibrosis, carcinogenesis, and metastasis [11]. The expression of some antioxidant enzymes is known to be controlled by the master transcription factor regulator Nrf2 [11,18]. The activation of Nrf2 involves a suppressor pro tein known as Kelch Like ECH Associated Protein 1 (Keap1) that binds Nrf2 in the cytoplasm, preventing its translocation into the nucleus for binding specific promoters [11,18].

Reactive oxygen species are known to alter the expression of several genes through induction of genetic mutations, resulting in alteration of the balance between cell proliferation and apoptosis [1,11,19]. Damage to DNA by ROS is now accepted as a major cause of cancer, and has been demonstrated in both breast and hepatocellular carcinoma [20]. Oxida tion of DNA bases, such as thymidine glycol, 5 hydroxymethyl 2′
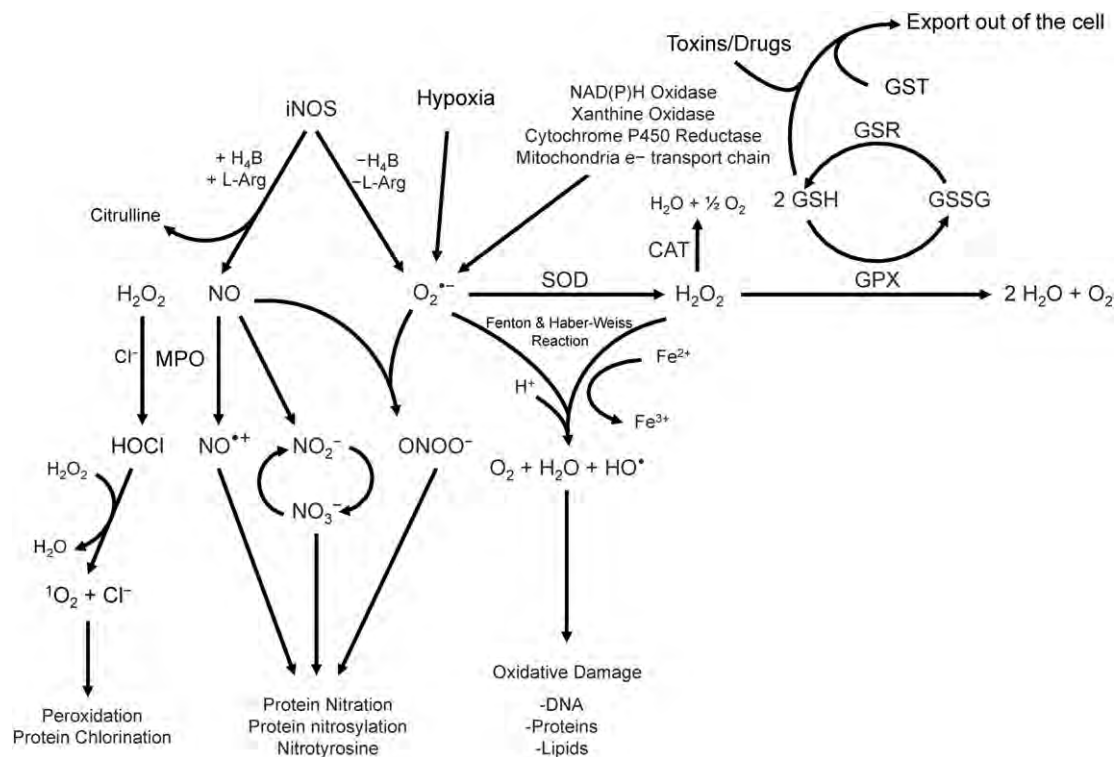
Fig. 1. Summary of key oxidant and antioxidants in cancer. Abbreviations are iNOS, inducible nitric oxide synthase; MPO, myeloperoxidase, H_2O_2, hydrogen peroxide; NO_2, nitrite; NO_3, nitrate; ONOO, peroxynitrite; L-Arg, L-arginine; HO•, hydroxyl radical; SOD, superoxide dismutase, CAT, catalase; GSH, glutathione; GPX, glutathione peroxidase; GSR, glutathione reductase; GST, glutathione S-transferase; GSSG, reduced glutathione; NAD(P)H, nicotinamide adenine dinucleotide phosphate; H_4B, tetrahydrobiopterin; Fe^{2+}, Iron (II); Fe^{3+}, Iron (III); O_2•, superoxide; NO•$^+$, nitrosonium cation; Cl, chloride ion; HOCl, hypochlorous acid.

deoxyuridine, and 8 OHdG are now considered as markers of DNA damage by oxidative stress [19]. More importantly, ROS are considered an essential factor in the maintenance of the oncogenic phenotype by activation of certain signaling pathways, specifically, the MAPK/AP 1 and NF κB pathways [20]. On the other hand, ROS are also required for the induction of cell death and thus can act as antitumor agents, which in this case is dependent on the concentration of ROS in the cellular environment [21].

Additionally, ROS are known to enhance tumor invasion and metastasis by increasing the rates of cell migration [1,11]. The NAD(P)H oxidase family of enzymes, a major source of cellular ROS, has been linked to the promotion of tumor cell survival and growth in pancreatic and lung cancers [1,11]. Reactive oxygen species regulate the expression of intercellular adhesion protein 1 (ICAM 1), a cell surface protein in endothelial and epithelial cells, through the activation of NF κB. ICAM 1 and IL 8 regulate the migration of neutrophils across the endothelium, which aid in tumor metastasis [11]. Another key player in the tumor invasion process is the upregulation of specific matrix metalloproteinases (MMPs), such as MMP 2, MMP 3, MMP 9, MMP 10, and MMP 13 by H_2O_2 and NO [11]. The mechanism of MMP upregulation involves the activation of Ras, the MAPK family members ERK1/2, p38, and JNK, or the inactivation of phosphatases [11,22]. Matrix metalloproteinases are essential enzymes in the degradation of most components of the basement membrane and extracellular matrix, such as type IV collagen [11,22].

Angiogenesis is critical for the survival of solid tumors and is also regulated by ROS [11]. Angiogenesis is regulated by a number of onco genes and tumor suppressor genes such as Ras, c Myc, c Jun, mutated p53, human epidermal growth factor receptor 2, and steroid receptor coactivators through the up regulation of VEGF or the down regulation of thrombospondin 1 (TSP 1), an angiogenesis suppressor [11]. Reactive oxygen species stabilizes HIF 1α protein and induces the production of angiogenic factors by tumor cells.

4. Cancer cells are under intrinsic oxidative stress

Cancer cells are known to manifest increased aerobic glycolysis (Warburg effect) and high levels of intrinsic oxidative stress [23,24]. Hypoxia triggers several critical adaptations that enable cell survival: it suppresses apoptosis, alters glucose metabolism, and triggers an angiogenic phenotype [15,23]. Recent investigations suggest that O_2 depletion stimulates mitochondria to produce ROS, which subsequently activates signaling pathways, such as HIF 1α, that promote cell survival and consequently, fibrotic growth [15]. Although HIF 1α is constitutively expressed, its half life is extremely short because it is rapidly hydroxylated by dioxygen, oxaloglutarate, and iron dependent prolyl 4 hydroxylases (PHD 1, 2, and 3), located in the nucleus, cytoplasm, or both, respectively [24,25]. Recent studies suggest that NO and ROS, some of which may be of mitochondrial origin, can promote HIF 1α stabilization by inhibiting (prolyl hydroxylase) PHD activity [15,26]. Super oxide is converted to H_2O_2 by SOD, and the resulting H_2O_2 efflux into the cytosol inhibits PHD activity, allowing HIF 1α to accumulate, dimerize with HIF 1β, and translocate into the nucleus where it modulates the expression of genes that favor survival under hypoxic conditions [15]. Support for the role of mitochondrial ROS in HIF 1α stabilization comes from studies showing that HIF 1α stabilization can be blocked under hypoxic conditions if ROS production is abrogated in mitochondria that lack cytochrome c or that have been treated with small interfering RNA (siRNA) to knock down the Rieske protein [15,27].

Several pro oxidant enzymes such as of myeloperoxidase (MPO), inducible nitric oxide synthase (iNOS) and NAD(P)H oxidase have been found in numerous types of malignant tumors including breast, lung, prostate, bladder, colorectal and malignant melanoma, while the expression strongly depends on the histological type/grade of the tumor [9,28,29]. Similarly, antioxidants have also been associated with cancer. Both GSR and GPX expression have been reported to be differentially expressed in various types of cancer [9]. Additionally, CAT was

G.M. Saed et al. / Gynecologic Oncology 145 (2017) 595–602

decreased in breast, bladder, and lung cancer while increased in brain cancer [9,28,29]. Superoxide dismutase is expressed in lung, colorectal, gastric, ovarian, and breast cancer, while decreased activity and expression have been reported in colorectal carcinomas and pancreatic cancer cells [9,28,29]. Collectively, this differential expression of oxidants and antioxidants demonstrates how the microenvironment of cancer is both unique and complex.

5. Ovarian cancer cells manifest a persistent pro-oxidant state

Oxidative stress has been implicated in the pathogenesis of several malignancies, including ovarian cancer [24,30]. Evidence suggests that ovarian cancer patients have decreased levels of circulating antioxidants and higher levels of oxidative stress [10,23,24,30 32]. In the past two decades, it has been reported that epithelial ovarian cancer (EOC) tissues and cells manifest a pro oxidant state characterized by an increased expression of key pro oxidant enzymes and decreased expression of antioxidant enzymes [31 33] (Table 1). Specifically, EOC cells and tissues manifested an increased expression of iNOS, MPO, NAD(P)H oxidase, as well as an increase in NO levels which correlated with expression in iNOS [31 33] (Table 1). Moreover, EOC cells manifested lower apoptosis, which was markedly induced by inhibiting iNOS with L NAME, indicating a strong link between apoptosis and the NO/iNOS pathways in these cells [33]. More importantly, it was found that EOC cells manifested a significant increase in S nitrosylation of caspase 3, which correlated with a significant decrease in caspase 3 activity, suggesting a potential mechanism of delayed apoptosis that was observed in these cells. Myeloperoxidase is a key oxidant enzyme that utilizes NO produced by iNOS, as a one electron substrate generating nitrosonium cation (NO^+), a labile nitrosating species [32,34,35]. Interestingly, MPO was only recently found to be expressed by EOC cells and tissues, and has since been confirmed by other investigators [10, 32,36]. Collectively, these findings suggests that MPO is a key player in regulating apoptosis in EOC cells, but also highlights a possible cross talk between iNOS and MPO in ovarian cancer [32].

Myeloperoxidase, an abundant hemoprotein previously known to be present solely in neutrophils and monocytes, plays an essential role in immune surveillance and host defense mechanisms, and can contribute to 3 nitrotyrosine formations in vivo and directly modulates inflammatory responses via regulation of NO bioavailability during inflammation [32,37]. Silencing MPO gene expression utilizing MPO specific siRNA induced apoptosis in EOC cells through a mechanism that involved the S nitrosylation of caspase 3 by MPO [32]. Additionally, MPO can serve as a source of free iron under oxidative stress, where both NO and $O_2\bullet^-$ are elevated [10,32]. Iron reacts with H_2O_2 and catalyzes the generation of highly reactive hydroxyl radical (HO•), thereby increasing oxidative stress, which in turn increases free iron concentrations by the Fenton and Haber Weiss reaction [10,32]. The potential

benefits of the combination of serum MPO and free iron as biomarkers for early detection of ovarian cancer have now been established [10]. Collectively, there is now substantial evidence demonstrating that altered oxidative stress may play a role in maintaining the oncogenic phenotype of ovarian cancer cells, and is summarized in Fig. 2.

6. Oxidative stress triggers cancer cells to favor anaerobic metabolism

Oxidative stress triggers cancer cells to favor anaerobic metabolism, despite the fact that oxygen is present [38,39]. This altered metabolism consists of an increase in glycolysis that is maintained in conditions of high oxygen tension ("aerobic glycolysis") and gives rise to enhanced lactate production [38 40]. To compensate for the reduction in cellular ATP production, [aerobic glucose oxidation generates more ATP per glucose molecule (36 ATP) as compared to glycolysis (2 ATP)], and cancer cells upregulate glucose receptors and significantly increase glucose uptake [24,25,40]. Aerobic glycolysis, in tumor cells, results in significant lactic acidosis, which additionally induces substantial toxicity to the surrounding tissues and in cancer cells themselves. Furthermore it has been shown that lactic acidosis facilitates tumor growth, in part through breakdown of extracellular matrix, increased cell mobility/metastatic potential, and activation of angiogenesis [40]. One of the foremost nearly ubiquitous mechanisms of aerobic glycolysis resides in the activation of HIF, an oxygen sensitive transcription factor that is activated by hypoxic stress as well as oncogenic, inflammatory, metabolic, and oxidative stress [40]. The link between oxidative stress and aerobic glycolysis is supported by the fact that HIF is activated under hypoxic conditions and is known to induce the expression of several glucose transporters as well as most of the enzymes required for glycolysis [41]. Hypoxia inducible factor also induces the expression of pyruvate dehydrogenate kinase (PDK), an enzyme that regulates the entry of pyruvate into the mitochondria [25,40,42]. Activated PDK can inhibit pyruvate dehydrogenase (PDH), thereby limiting the entry of pyruvate into the mitochondria, where glucose oxidation can occur.

Dichloroacetate (DCA) is a metabolic modulator that has been clinically utilized in the treatment of hereditary mitochondrial diseases as well as lactic acidosis [25,43]. Dichloroacetate inhibits PDK and thus shifts glucose metabolism in cancer cells from glycolysis to glucose oxidation, reversing the unique aerobic glycolysis found in solid tumors [44]. Consistent with these findings, DCA treatment significantly decreased HIF 1α expression [24]. Dichloroacetate has been shown to shift the oxidative balance in the intracellular redox state, leading to the activation of specific endonucleases, which induce apoptosis in EOC cells [24]. Treatment of EOC cells with DCA significantly induced apoptosis through the stimulation of caspase 3 activity in a dose dependent manner, and was confirmed by the TUNEL assay [24]. Indeed, DCA has also been shown to induce apoptosis in cancer cells as evident by the efflux of cytochrome c and apoptosis inducing factor from the mitochondria [45]. In support of these findings, it has been shown that aerobic glycolysis, as a result of oxidative stress, can result in resistance to apoptosis [24,46]. Several enzymes involved in glycolysis are also known to regulate apoptosis and gene transcription, suggesting that links between metabolic sensors, cell death, and gene transcription are established directly through the enzymes that control metabolism [25, 47]. Additionally, DCA induces apoptosis in glioblastoma, endometrial, prostate, and nonsmall cell lung cancers, further supporting the findings from this study, which aimed to establish a link between DCA, oxidative stress, and apoptosis in EOC cell lines, possibly through similar mechanisms [25].

Since DCA acts by activating PDH, through the inhibition of PDK, bringing pyruvate into the mitochondria and enhancing glucose oxidation, it is therefore an ideal approach to shift aerobic glycolysis to glucose oxidation coupling rather than just inhibiting aerobic glycolysis. Inhibiting aerobic glycolysis results in ATP depletion and necrosis, not apoptosis, because apoptosis is an energy consuming process, requiring

Table 1
Summary of oxidant and antioxidant expression in sensitive and chemoresistant ovarian cancer. Abbreviations are iNOS, inducible nitric oxide synthase; MPO, myeloperoxidase; SOD, superoxide dismutase, CAT, catalase; GSH, glutathione; GPX, glutathione peroxidase; GSR, glutathione reductase; NAD(P)H, nicotinamide adenine dinucleotide phosphate.

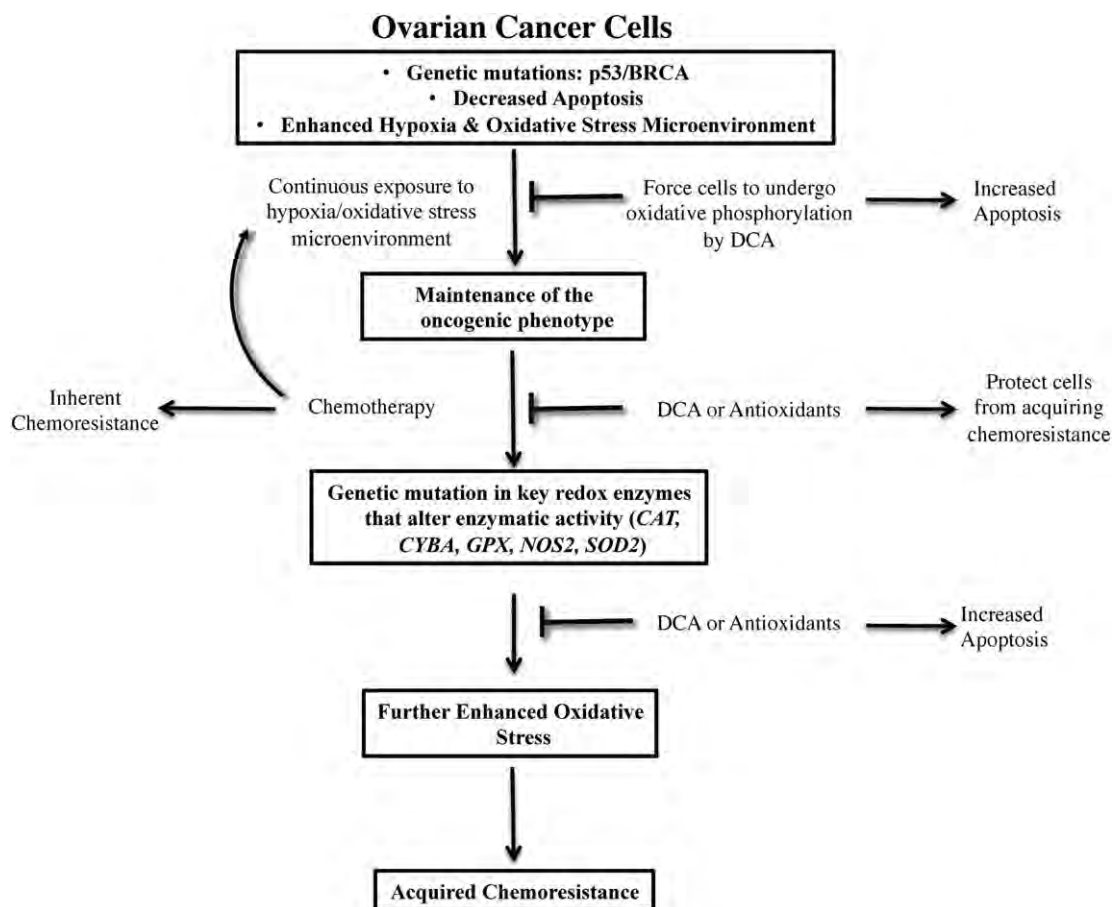|	Ovarian cancer	Chemoresistant ovarian cancer	Reference
Oxidants			
MPO	↑	↑↑	[10,32,36]
iNOS	↑	↑↑	[28,32]
Nitrite/nitrate		↑↑	[9,28]
NAD(P)H oxidase	↑		[31]
Antioxidants			
CAT	↓	↑↑	[9,30]
GSH	↑↑	↑↑	[58]
GSR		↓↓	[9,28]
GPX		↑↑	[9]
SOD	↓	↓↓	[9,30]

Ovarian Cancer Cells

Fig. 2. Summary of the role of oxidative stress in the development of sensitive and chemoresistant ovarian cancer.

active mitochondria [25,48]. Dichloroacetate activates PDH through the inhibition of PDK at concentrations of 10 to 250 mmol/L or 0.15 to 37.5 mg/mL, in a dose dependent fashion [25,49]. Four different iso forms of PDK have been identified that have variable expression and sensitivity to the inhibition by DCA [25,50]. Moreover, DCA adminis tered at 35 to 50 mg/kg decreases lactate levels by more than 60% and directly activates PDH by 3 to 6 fold [25,49].

The high levels of ROS and RNS manifested by tumor cells can be countered by high levels of antioxidants, such as SOD [51]. Superoxide dismutase is considered a key antioxidant in aerobic cells and is respon sible for the elimination of $O_2\bullet^-$ by converting it to H_2O_2. Indeed, defi ciency in SOD or inhibition of the enzyme activity may cause accumulation of $O_2\bullet^-$ in the cells, which may result in the persistence of the oncogenic phenotype [52]. Interestingly, DCA has been shown to significantly induce the expression of SOD3 in EOC cells, however, in other studies using different cancer cell lines, it was reported that de creased levels of SOD are effective in the induction of apoptosis [23,24, 53]. Decreased levels of SOD may result in toxic high levels of free radi cals, which ultimately could lead to necrosis. On the other hand, ROS can also induce cellular senescence and cell death and can therefore func tion as antitumorigenic agents [24,54]. Whether ROS promote tumor cell survival or act as antitumorigenic agents depends on the cell and tis sues, the location of ROS production, and the concentration of individual ROS [11].

In summary, studies have shown that shifting anaerobic to aerobic metabolism by DCA induces apoptosis of EOC cells [24]. This effect was attributed to the modulation of key enzymes that are central to con trolling the cellular redox balance. The utilization of DCA to induce apo ptosis of EOC cells may provide a therapeutic option in the treatment of EOC. Explicably, the potential therapeutic value of DCA for ovarian can cer will require future analysis utilizing more cell lines, including

ovarian surface epithelial cells, fallopian tube secretory epithelial cells, as well as patients.

7. Chemotherapy and the acquisition of chemoresistance in EOC cells

Despite significant advances in surgery and anticancer treatment, chemotherapy resistance remains a major obstacle to improving a can cer patient's outcome [55]. Taxanes and platinums are the current drug therapies used for treatment of ovarian cancer. Chemoresistance greatly limits the range of possibilities for subsequent treatments, because some tumors become resistant not only to the initial drug but also to new therapeutic agents with different mechanisms of action [56]. Many chemotherapy drugs serve as a source of oxidative stress through a direct mechanism of cell death, or as an indirect effect of exposure, as observed with several chemotherapeutic agents [57]. Known factors af fecting the occurrence of resistance include: altered drug influx/efflux, increased cellular GSH levels, upregulation of *Bcl 2*, decreased platinum accumulation in tumor cells, increased GSH synthesis, loss of tumor ne crosis factor receptor apoptosis inducing ligand (TRAIL) induced apo ptosis, increased DNA repair and enhanced ability to repair through up regulation of DNA repair genes [11]. Moreover, overexpression of GST is known to reduce the reactivity of various chemotherapy drugs [58]. Additionally, loss of functional p53 augments NF κB activated in flammation, thus, stabilization of wild type p53 is critical for the pre vention of EOC from progression to drug resistance [11]. Chemoresistant EOC cells have been shown to exhibit increased expres sion of iNOS and nitrate/nitrite levels as well as a decrease in GSR ex pression, suggesting a shift towards a severe pro oxidant state by these cells [28] (Table 1).

As mentioned earlier, EOC cells are known to manifest a pro oxidant state characterized by increased key oxidant enzymes with concomitant decreased antioxidant enzymes [28] (Table 1). Chemotherapy resistant EOC cells are now known to also manifest an alteration in the redox balance, further advancing this pro oxidant environment [9]. Indeed, there was a significant increase in levels of CAT, GPX, and iNOS in chemoresistant EOC cells as compared to their sensitive counterparts [9] (Table 1). In contrast, there was a decrease in levels in GSR, SOD, and the NAD(P)H oxidase subunit (p22^{phox}) in chemoresistant EOC cells [9]. This data supports an important role for an altered redox balance, not only in the maintenance of the oncogenic phenotype, but also in the development of chemoresistance (Fig. 2).

8. Polymorphisms in key oxidant and antioxidant enzymes are associated with ovarian cancer

A single nucleotide polymorphism (SNP) occurs because of point mutations that are selectively maintained in populations and are distributed throughout the human genome at an estimated overall frequency of at least one in every 1000 base pairs [59]. Recent evidence demonstrates an association between enzymatic activity altering SNPs in oxidative DNA repair genes and antioxidant genes with human cancer susceptibility [13]. Additionally, a pro oxidant state has been implicated in the pathogenesis of several malignancies, including ovarian cancer [24,31]. This area of research is essentially reorganizing our understanding of inheritance and evolution. These modifications might explain the in vitro persistence of the oncogenic phenotype even after normal conditions are restored, as well as the clinical propensity for individuals to develop cancer.

This mechanism of altered enzymatic activity further explains the observation of significantly decreased apoptosis and increased survival of EOC cells [32]. Investigations into the effect of SNPs on various redox enzymes are an active area of scientific research [9,29,60,61]. The effects of genetic polymorphisms in oxidative stress related genes on cancer susceptibility may be determined by studying functional polymorphisms in genes that control the levels of cellular ROS and oxidative damage, including SNPs for genes involved in carcinogen metabolism (detoxification and/or activation), antioxidants, and DNA repair pathways [60]. Several SNPs have been identified in key antioxidants, leading to change of function, including *CAT*, *GPX1*, *GSR*, and *SOD2* [9,61]. In support of this, recent studies have also associated genetic polymorphisms in genes involved in suppression of tumorigenicity as well as those involved in cell cycle with ovarian cancer [62,63]. Additional genetic variations, many of which have been identified in recent genome wide association studies (GWAS), have been hypothesized to act as low to moderate penetrant alleles, which contribute to ovarian cancer risk, as well as other diseases [7,64].

There now is convincing evidence to suggest an association of specific SNPs in key redox enzymes with increased risk and overall survival of ovarian cancer [9,29]. Recently, a specific *CAT* SNP (rs1001179), that leads to reduced enzyme activity, was reported to be associated with increased risk for breast cancer and has also been described to be a significant predictor of death when present in ovarian cancer patients [9,29,61,65]. This finding is consistent with several other studies, which linked this specific SNP with risk, response to adjuvant treatment and survival of cancer patients, including ovarian [29,66].

NAD(P)H oxidase, a key pro oxidant enzyme, is a significant source of ROS. The membrane bound components of NAD(P)H oxidase are the catalytic subunit *CYBB* (gp91^{phox}) and the adjacent oxygen sensing subunit *CYBA* (p22^{phox}) [9,29]. Several SNPs for CYBA have been reported, some of which alter the enzyme activity. A specific SNP in CYBA (rs4673) was associated with an increased risk for ovarian cancer and other diseases where oxidative stress plays a critical role in their pathophysiology, including cardiovascular disease, asthma, and diabetic nephropathy [9,29]. The mutant genotype of the *CYBA* gene has been

shown to both decrease and increase activity of the protein, thereby altering the generation of $O_2^{\bullet-}$ [9,29].

Recent genetic studies have linked MPO to lung and ovarian cancers by demonstrating a striking correlation between the relative risk for development of the disease and the incidence of functionally distinct *MPO* polymorphisms [9,29]. Specifically, a SNP in *MPO* (rs2333227) was shown to be associated with increased risk for ovarian cancer [36]. Genome wide association studies have also successfully identified and confirmed six SNPs that appear to influence the risk of EOC [9,29]. The confirmed susceptibility SNPs are rs3814113 (located at 9p22, near BNC2), rs2072590 (located at 2q31, which contains a family of HOX genes), rs2665390 (located at 3q25, intronic to TIPARP), rs10088218 (located at 8q24, 700 kb downstream of MYC), rs8170 (located at 19p13, near MERIT40), and rs9303542 (located at 17q21, intronic to SKAP1) [9,29]. Therefore, some believe that the genetic component of ovarian cancer risk may be attributed to genetic polymorphisms that confer low to moderate risk, such as SNPs that result in point mutations in the gene [67].

9. Acquisition of chemoresistance in ovarian cancer cells is associated with specific point mutations in key redox enzymes

The mechanisms underlying the acquisition of chemoresistance in ovarian cancer have yet to be fully elucidated. Evidence for an enhanced pro oxidant state in chemoresistant EOC cells has now been described, and is thought to be a result of point mutations in key redox enzymes [9]. Specifically, a recent study observed a significant increase in levels of CAT, GPX, and iNOS while there was a significant decrease in levels of GSR, SOD, and NAD(P)H oxidase in chemoresistant EOC cells as compared to their sensitive counterparts [9]. These findings suggest a role for an altered redox balance in the development of chemoresistance in ovarian cancer. To investigate a possible mechanism of altered redox enzyme levels, the presence of several SNPs was determined in both sensitive and chemoresistant EOC cell lines. Indeed, docetaxel and/or cisplatin chemoresistant EOC cells were characterized to manifest specific point mutations, corresponding to known functional SNPs, in key redox enzymes including *SOD2* (rs4880), *NOS2* (rs2297518), and *CYBA* (rs4673) which are not present in their sensitive counterparts (Table 1). Interestingly, chemoresistant EOC cells exhibited an altered enzymatic activity for CAT and GSR while they did not exhibit the specific SNP of interest in those enzymes, which again suggests possible involvement in other functional SNPs for those enzymes (Table 1) [9]. The fact that the SNP was present in the chemoresistant EOC cells and not the sensitive cell line from with it was derived suggests that in fact, this is a point mutation rather than a SNP. To determine whether chemotherapy was capable of inducing point mutations that happen to correspond to known functional SNPs, specific point mutations in *SOD2* or *GPX1* were induced in sensitive EOC cells which led to a decrease in the sensitivity to chemotherapy, suggesting acquisition of chemoresistance [9]. Furthermore, treatment of sensitive and chemoresistant EOC cells with SOD combined with chemotherapy significantly increased the efficacy of the chemotherapy in a synergistic manner, with a more drastic effect in the chemoresistant cells [9]. This observation suggests that induction of specific point mutations in sensitive EOC cells corresponding to functional SNPs found in chemoresistant EOC cells directly reduced the sensitivity to chemotherapy (Fig. 2). These findings also support the notion that chemotherapy can induce gene point mutations that happen to correspond to SNPs in locations with functional effects, thus altering overall redox balance for survival (Fig. 2) [9].

One possible explanation for the observed nucleotide switches in response to chemotherapy is nucleotide substitution, a mechanism which includes transitions, replacement of one purine by the other or that of one pyrimidine by the other, or transversions, replacement of a purine by a pyrimidine or vice versa [9]. It has been established that hydroxyl radicals react with DNA causing the formation of a large number of

pyrimidine and purine derived lesions [9]. The oxidative damage to 8 Oxo 2′ deoxyguanosine, an oxidized derivative of deoxyguanosine and major product of DNA oxidation, has been implicated in tumor initiation and progression through accumulation of genetic alterations of both oncogenes and tumor suppressor genes [9]. Indeed, previous findings revealed that GC → TA transversions derived from 8 hydroxy 2′ deoxyguanosine have been reported in the *ras* oncogene and the *p53* tumor suppressor gene in several cancers. It should be indicated however that GC → TA transversions are not unique to hydroxy 2′ deoxyguanosine, CC → TT substitutions have been identified as signature mutations for ROS [9].

Another explanation for the nucleotide switch is that chemoresistance resulted in an entirely different population of cells, with a new genotype. Chemotherapy eliminates the bulk of the tumor while leaving a core of cancer cells with high capacity for repair and renewal, known as cancer stem cells (CSCs) [9]. Tumors arising from CSCs usually contain a mixed population of cells due to the property of asymmetric division [9]. Cancer stem cells have been isolated from various types of cancer including leukemia, breast, brain, pancreatic, prostate, ovarian and colon [9]. Strikingly, CSCs were reported to be present in SKOV 3 EOC cells [9]. Additionally, CSCs have been shown to confer chemoresistance to cisplatin and doxorubicin in ovarian cancer cells [9].

10. Ovarian cancer immunotherapy and oxidative stress

It is well established that tumorigenic cells generate high levels of ROS to activate proximal signaling pathways that promote proliferation, survival and metabolic adaptation while also maintaining a high level of antioxidant activity to prevent buildup of ROS to levels that could induce cell death [68]. Moreover, there is evidence that ROS can act as second messengers in immune cells, which can lead to hyperactivation of inflammatory responses resulting in tissue damage and pathology [68]. Ovarian cancer is considered an ideal tumorigenic cancer because ovarian cancer cells have no negative impact on immune cells [69].

Effective immunotherapy for ovarian cancer is currently the focus of several investigations and clinical trials. Current immunotherapies for cancer treatment include therapeutic vaccines, cytokines, immune modulators, immune checkpoint inhibitors, and adoptive T cell transfer [70]. The discovery of a monoclonal antibody (bevacizumab) directed against vascular endothelial growth factor (VEGF) which has been shown to improve progression free survival compared to cytotoxic chemotherapy alone was a major outcome of clinical trials [71]. Other monoclonal antibodies currently approved for other cancers such as trastuzumab for breast cancer or cetuximab for colon cancer exhibited limited activity in ovarian cancer [71]. Several clinical trials are ongoing for the utilization of immune checkpoint blockade in ovarian cancer immune therapy [72]. Most recently tested were the programmed death (PD) 1 inhibitors, pembrolizumab and nivolumab, which showed a consistent response rate of 10 20% in phase 2 studies and then failed to improve outcomes in confirmatory trials [72]. Ultimately, larger phase 3 studies are needed to validate these findings for checkpoint inhibitors, particularly with regard to the duration of response seen with these agents. Additionally, the direct intraperitoneal delivery of interleukin (IL) 12, a potent immunostimulatory agent, exhibited some potential therapeutic efficacy in ovarian cancer [73]. Recently, targeting folate receptor alpha, which is found to be expressed in ovarian cancer, has shown promising therapeutic value. The targeting of the folate receptor was achieved by either a blocking monoclonal antibody (farletuzumab) or antibody conjugates of folate analogs, such as vintafolide [74].

11. Summary and conclusion

Oxidative stress has been implicated in the pathogenesis of several malignancies including ovarian cancer. Epithelial ovarian cancer is characterized to manifest a persistent pro oxidant state through alteration of the redox balance, which is further enhanced in their chemoresistant counterparts, as summarized in Table 1 and Fig. 2. Forcing ovarian cancer cells to undergo oxidative phosphorylation rather than glycolysis has been shown to be beneficial for eliminating cells via apoptosis (Fig. 2). Collectively, there is convincing evidence that indicated a causal relationship between the acquisition of chemoresistance and chemotherapy induced genetic mutations in key redox enzymes, leading to a further enhanced oxidative stress in chemoresistant EOC cells. This concept was further confirmed by the observation that induction of point mutations in sensitive EOC cells increased their resistance to chemotherapy. Also, a combination of antioxidants with chemotherapy significantly sensitized cells to chemotherapy. Identification of targets for chemoresistance with either biomarker and/or screening potential will have a significant impact for the treatment of this disease.

Conflicts of interest

References

[1] V. Rojas, K.M. Hirshfield, S. Ganesan, L. Rodriguez-Rodriguez, Molecular characterization of epithelial ovarian cancer: implications for diagnosis and treatment, Int. J. Mol. Sci. 17 (2016).

[2] B.K. Erickson, M.G. Conner, C.N. Landen Jr., The role of the fallopian tube in the origin of ovarian cancer, Am. J. Obstet. Gynecol. 209 (2013) 409–414.

[3] R.J. Kurman, Ie M. Shih, The dualistic model of ovarian carcinogenesis: revisited, revised, and expanded, Am. J. Pathol. 186 (2016) 733–747.

[4] J. Liao, F. Qian, N. Tchabo, P. Mhawech-Fauceglia, A. Beck, Z. Qian, et al., Ovarian cancer spheroid cells with stem cell-like properties contribute to tumor generation, metastasis and chemotherapy resistance through hypoxia-resistant metabolism, PLoS One 9 (2014), e84941.

[5] E. Lengyel, Ovarian cancer development and metastasis, Am. J. Pathol. 177 (2010) 1053–1064.

[6] K.A. Vermeersch, L. Wang, R. Mezencev, J.F. McDonald, M.P. Styczynski, OVCAR-3 spheroid-derived cells display distinct metabolic profiles, PLoS One 10 (2015), e0118262.

[7] S.J. Ramus, R.A. Vierkant, S.E. Johnatty, M.C. Pike, D.J. Van Den Berg, A.H. Wu, et al., Consortium analysis of 7 candidate SNPs for ovarian cancer, Int. J. Cancer 123 (2008) 380–388.

[8] K. Hibbs, K.M. Skubitz, S.E. Pambuccian, R.C. Casey, K.M. Burleson, T.R. Oegema Jr., et al., Differential gene expression in ovarian carcinoma: identification of potential biomarkers, Am. J. Pathol. 165 (2004) 397–414.

[9] N.M. Fletcher, J. Belotte, M.G. Saed, I. Memaj, M.P. Diamond, R.T. Morris, et al., Specific point mutations in key redox enzymes are associated with chemoresistance in epithelial ovarian cancer, Free Radic. Biol. Med. 102 (2016) 122–132.

[10] N.M. Fletcher, Z. Jiang, R. Ali-Fehmi, N.K. Levin, J. Belotte, M.A. Tainsky, et al., Myeloperoxidase and free iron levels: potential biomarkers for early detection and prognosis of ovarian cancer, Cancer Biomark. 10 (2011) 267–275.

[11] S. Reuter, S.C. Gupta, M.M. Chaturvedi, B.B. Aggarwal, Oxidative stress, inflammation, and cancer: how are they linked? Free Radic. Biol. Med. 49 (2010) 1603–1616.

[12] X.G. Lei, J.H. Zhu, W.H. Cheng, Y. Bao, Y.S. Ho, A.R. Reddi, et al., Paradoxical roles of antioxidant enzymes: basic mechanisms and health implications, Physiol. Rev. 96 (2016) 307–364.

[13] J.E. Klaunig, L.M. Kamendulis, B.A. Hocevar, Oxidative stress and oxidative damage in carcinogenesis, Toxicol. Pathol. 38 (2010) 96–109.

[14] L.M. Coussens, Z. Werb, Inflammation and cancer, Nature 420 (2002) 860–867.

[15] J.P. Fruehauf, F.L. Meyskens Jr., Reactive oxygen species: a breath of life or death? Clin. Cancer Res. 13 (2007) 789–794.

[16] M.L. Circu, T.Y. Aw, Glutathione and modulation of cell apoptosis, Biochim. Biophys. Acta 2012 (1823) 1767–1777.

[17] T. Ishikawa, F. Ali-Osman, Glutathione-associated cis-diamminedichloroplatinum(II) metabolism and ATP-dependent efflux from leukemia cells. Molecular characterization of glutathione-platinum complex and its biological significance, J. Biol. Chem. 268 (1993) 20116–20125.

[18] H.H. Schmidt, R. Stocker, C. Vollbracht, G. Paulsen, D. Riley, A. Daiber, et al., Antioxidants in translational medicine, Antioxid. Redox Signal. 23 (2015) 1130–1143.

[19] W.P. Roos, A.D. Thomas, B. Kaina, DNA damage and the balance between survival and death in cancer biology, Nat. Rev. Cancer 16 (2016) 20–33.

[20] G. Waris, H. Ahsan, Reactive oxygen species: role in the development of cancer and various chronic conditions, J. Carcinog. 5 (2006) 14.

[21] J. Wang, J. Yi, Cancer cell killing via ROS: to increase or decrease, that is the question, Cancer Biol. Ther. 7 (2008) 1875–1884.

[22] J. Westermarck, V.M. Kahari, Regulation of matrix metalloproteinase expression in tumor invasion, FASEB J. 13 (1999) 781–792.

[23] E.O. Hileman, J. Liu, M. Albitar, M.J. Keating, P. Huang, Intrinsic oxidative stress in cancer cells: a biochemical basis for therapeutic selectivity, Cancer Chemother. Pharmacol. 53 (2004) 209–219.

[24] G.M. Saed, N.M. Fletcher, Z.L. Jiang, H.M. Abu-Soud, M.P. Diamond, Dichloroacetate induces apoptosis of epithelial ovarian cancer cells through a mechanism involving modulation of oxidative stress, Reprod. Sci. 18 (2011) 1253–1261.

[25] E.D. Michelakis, L. Webster, J.R. Mackey, Dichloroacetate (DCA) as a potential metabolic-targeting therapy for cancer, Br. J. Cancer 99 (2008) 989–994.

[26] E.L. Bell, B.M. Emerling, N.S. Chandel, Mitochondrial regulation of oxygen sensing, Mitochondrion 5 (2005) 322–332.

[27] K.D. Mansfield, R.D. Guzy, Y. Pan, R.M. Young, T.P. Cash, P.T. Schumacker, et al., Mitochondrial dysfunction resulting from loss of cytochrome c impairs cellular oxygen sensing and hypoxic HIF-alpha activation, Cell Metab. 1 (2005) 393–399.

[28] J. Belotte, N.M. Fletcher, A.O. Awonuga, M. Alexis, H.M. Abu-Soud, M.G. Saed, et al., The role of oxidative stress in the development of cisplatin resistance in epithelial ovarian cancer, Reprod. Sci. 21 (2014) 503–508.

[29] J. Belotte, N.M. Fletcher, M.G. Saed, M.S. Abusamaan, G. Dyson, M.P. Diamond, et al., A single nucleotide polymorphism in catalase is strongly associated with ovarian cancer survival, PLoS One 10 (2015), e0135739.

[30] K. Senthil, S. Aranganathan, N. Nalini, Evidence of oxidative stress in the circulation of ovarian cancer patients, Clin. Chim. Acta 339 (2004) 27–32.

[31] Z. Jiang, N.M. Fletcher, R. Ali-Fehmi, M.P. Diamond, H.M. Abu-Soud, A.R. Munkarah, et al., Modulation of redox signaling promotes apoptosis in epithelial ovarian cancer cells, Gynecol. Oncol. 122 (2011) 418–423.

[32] G.M. Saed, R. Ali-Fehmi, Z.L. Jiang, N.M. Fletcher, M.P. Diamond, H.M. Abu-Soud, et al., Myeloperoxidase serves as a redox switch that regulates apoptosis in epithelial ovarian cancer, Gynecol. Oncol. 116 (2010) 276–281.

[33] J.M. Malone, G.M. Saed, M.P. Diamond, R.J. Sokol, A.R. Munkarah, The effects of the inhibition of inducible nitric oxide synthase on angiogenesis of epithelial ovarian cancer, Am. J. Obstet. Gynecol. 194 (2006) 1110–1116 (discussion 6-8).

[34] H.M. Abu-Soud, S.L. Hazen, Nitric oxide is a physiological substrate for mammalian peroxidases, J. Biol. Chem. 275 (2000) 37524–37532.

[35] H.M. Abu-Soud, S.L. Hazen, Nitric oxide modulates the catalytic activity of myeloperoxidase, J. Biol. Chem. 275 (2000) 5425–5430.

[36] D.C. Castillo-Tong, D. Pils, G. Heinze, I. Braicu, J. Sehouli, A. Reinthaller, et al., Association of myeloperoxidase with ovarian cancer, Tumour Biol. 35 (2014) 141–148.

[37] S. Galijasevic, G.M. Saed, S.L. Hazen, H.M. Abu-Soud, Myeloperoxidase metabolizes thiocyanate in a reaction driven by nitric oxide, Biochemistry 45 (2006) 1255–1262.

[38] B. Chen, H. Li, X. Zeng, P. Yang, X. Liu, X. Zhao, et al., Roles of microRNA on cancer cell metabolism, J. Transl. Med. 10 (2012) 228.

[39] M.G. Vander Heiden, J.W. Locasale, K.D. Swanson, H. Sharfi, G.J. Heffron, D. Amador-Noguez, et al., Evidence for an alternative glycolytic pathway in rapidly proliferating cells, Science (New York, N.Y.) 329 (2010) 1492–1499.

[40] G. Kroemer, J. Pouyssegur, Tumor cell metabolism: cancer's Achilles' heel, Cancer Cell 13 (2008) 472–482.

[41] G.L. Semenza, P.H. Roth, H.M. Fang, G.L. Wang, Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor 1, J. Biol. Chem. 269 (1994) 23757–23763.

[42] J.W. Kim, I. Tchernyshyov, G.L. Semenza, C.V. Dang, HIF-1-mediated expression of pyruvate dehydrogenase kinase: a metabolic switch required for cellular adaptation to hypoxia, Cell Metab. 3 (2006) 177–185.

[43] P.W. Stacpoole, L.R. Gilbert, R.E. Neiberger, P.R. Carney, E. Valenstein, D.W. Theriaque, et al., Evaluation of long-term treatment of children with congenital lactic acidosis with dichloroacetate, Pediatrics 121 (2008) e1223–e1228.

[44] J. Xie, B.S. Wang, D.H. Yu, Q. Lu, J. Ma, H. Qi, et al., Dichloroacetate shifts the metabolism from glycolysis to glucose oxidation and exhibits synergistic growth inhibition with cisplatin in HeLa cells, Int. J. Oncol. 38 (2011) 409–417.

[45] L.H. Stockwin, S.X. Yu, S. Borgel, C. Hancock, T.L. Wolfe, L.R. Phillips, et al., Sodium dichloroacetate selectively targets cells with defects in the mitochondrial ETC, Int. J. Cancer 127 (2010) 2510–2519.

[46] J.W. Kim, C.V. Dang, Cancer's molecular sweet tooth and the Warburg effect, Cancer Res. 66 (2006) 8927–8930.

[47] J.W. Kim, C.V. Dang, Multifaceted roles of glycolytic enzymes, Trends Biochem. Sci. 30 (2005) 142–150.

[48] R.H. Xu, H. Pelicano, Y. Zhou, J.S. Carew, L. Feng, K.N. Bhalla, et al., Inhibition of glycolysis in cancer cells: a novel strategy to overcome drug resistance associated with mitochondrial respiratory defect and hypoxia, Cancer Res. 65 (2005) 613–621.

[49] P.W. Stacpoole, The pharmacology of dichloroacetate, Metabolism 38 (1989) 1124–1144.

[50] M.C. Sugden, M.J. Holness, Recent advances in mechanisms regulating glucose oxidation at the level of the pyruvate dehydrogenase complex by PDKs, Am. J. Physiol. Endocrinol. Metab. 284 (2003) E855–E862.

[51] V.L. Kinnula, J.D. Crapo, Superoxide dismutases in malignant cells and human tumors, Free Radic. Biol. Med. 36 (2004) 718–744.

[52] R. Tandon, M.K. Sinha, H. Garg, R. Khanna, H.D. Khanna, Oxidative stress in patients with essential hypertension, Natl Med. J. India 18 (2005) 297–299.

[53] P. Huang, L. Feng, E.A. Oldham, M.J. Keating, W. Plunkett, Superoxide dismutase as a target for the selective killing of cancer cells, Nature 407 (2000) 390–395.

[54] P. Storz, Reactive oxygen species in tumor progression, Front. Biosci. 10 (2005) 1881–1896.

[55] C. Lee, P. Macgregor, Drug resistance and microarrays, Mod. Drug Discovery 7 (2004).

[56] H. Kajiyama, K. Shibata, M. Mizuno, T. Umezu, S. Suzuki, R. Sekiya, et al., Survival benefit of taxane plus platinum in recurrent ovarian cancer with non-clear cell, non-mucinous histology, J. Gynecol. Oncol. 25 (2014) 43–50.

[57] M. Landriscina, F. Maddalena, G. Laudiero, F. Esposito, Adaptation to oxidative stress, chemoresistance, and cell survival, Antioxid. Redox Signal. 11 (2009) 2701–2716.

[58] N. Traverso, R. Ricciarelli, M. Nitti, B. Marengo, A.L. Furfaro, M.A. Pronzato, et al., Role of glutathione in cancer progression and chemoresistance, Oxidative Med. Cell. Longev. 2013 (2013) 972913.

[59] H.C. Erichsen, S.J. Chanock, SNPs in cancer research and treatment, Br. J. Cancer 90 (2004) 747–751.

[60] J.E. Klaunig, Z. Wang, X. Pu, S. Zhou, Oxidative stress and oxidative damage in chemical carcinogenesis, Toxicol. Appl. Pharmacol. 254 (2011) 86–99.

[61] L. Forsberg, L. Lyrenas, U. de Faire, R. Morgenstern, A common functional C-T substitution polymorphism in the promoter region of the human catalase gene influences transcription factor binding, reporter gene transcription and is correlated to blood catalase levels, Free Radic. Biol. Med. 30 (2001) 500–505.

[62] E.L. Goode, B.L. Fridley, R.A. Vierkant, J.M. Cunningham, C.M. Phelan, S. Anderson, et al., Candidate gene analysis using imputed genotypes: cell cycle single-nucleotide polymorphisms and ovarian cancer risk, Cancer Epidemiol. Biomark. Prev. 18 (2009) 935–944.

[63] M. Notaridou, L. Quaye, D. Dafou, C. Jones, H. Song, E. Hogdall, et al., Common alleles in candidate susceptibility genes associated with risk and development of epithelial ovarian cancer, Int. J. Cancer 128 (2011) 2063–2074.

[64] S. Savas, S. Schmidt, H. Jarjanazi, H. Ozcelik, Functional nsSNPs from carcinogenesis-related genes expressed in breast tissue: potential breast cancer risk alleles and their distribution across human populations, Hum Genomics 2 (2006) 287–296.

[65] S.K. Quick, P.G. Shields, J. Nie, M.E. Platek, S.E. McCann, A.D. Hutson, et al., Effect modification by catalase genotype suggests a role for oxidative stress in the association of hormone replacement therapy with postmenopausal breast cancer risk, Cancer Epidemiol. Biomark. Prev. 17 (2008) 1082–1087.

[66] J. Didziapetriene, J. Bublevic, G. Smailyte, B. Kazbariene, R. Stukas, Significance of blood serum catalase activity and malondialdehyde level for survival prognosis of ovarian cancer patients, Medicina 50 (2014) 204–208.

[67] T.A. Sellers, Y. Huang, J. Cunningham, E.L. Goode, R. Sutphen, R.A. Vierkant, et al., Association of single nucleotide polymorphisms in glycosylation genes with risk of epithelial ovarian cancer, Cancer Epidemiol. Biomark. Prev. 17 (2008) 397–404.

[68] M. Schieber, N.S. Chandel, ROS function in redox signaling and oxidative stress, Curr. Biol. 24 (2014) R453–R462.

[69] L.E. Kandalaft, D.J. Powell Jr., N. Singh, G. Coukos, Immunotherapy for ovarian cancer: what's next? J. Clin. Oncol. 29 (2011) 925–933.

[70] F. De Felice, C. Marchetti, I. Palaia, D. Musio, L. Muzii, V. Tombolini, et al., Immunotherapy of ovarian cancer: the role of checkpoint inhibitors, J. Immunol. Res. 2015 (2015) 191832.

[71] B. Zand, R.L. Coleman, A.K. Sood, Targeting angiogenesis in gynecologic cancers, Hematol. Oncol. Clin. North Am. 26 (2012) 543–563 (viii).

[72] C. Chester, O. Dorigo, J.S. Berek, H. Kohrt, Immunotherapeutic approaches to ovarian cancer treatment, J. Immunother. Cancer 3 (2015) 7.

[73] R.D. Alvarez, M.W. Sill, S.A. Davidson, C.Y. Muller, D.P. Bender, R.L. DeBernardo, et al., A phase II trial of intraperitoneal EGEN-001, an IL-12 plasmid formulated with PEG-PEI-cholesterol lipopolymer in the treatment of persistent or recurrent epithelial ovarian, fallopian tube or primary peritoneal cancer: a gynecologic oncology group study, Gynecol. Oncol. 133 (2014) 433–438.

[74] C. Marchetti, I. Palaia, M. Giorgini, C. De Medici, R. Iadarola, L. Vertechy, et al., Targeted drug delivery via folate receptors in recurrent ovarian cancer: a review, OncoTargets Ther. 7 (2014) 1223–1236.

Exhibit 80

Inflammation: A hidden path to breaking the spell of ovarian cancer

Weiwei Shan & Jinsong Liu

REVIEW

Inflammation
A hidden path to breaking the spell of ovarian cancer

Weiwei Shan and Jinsong Liu*

Department of Pathology; The University of Texas M D Anderson Cancer Center; Houston TX USA

Epithelial ovarian cancer is a highly lethal gynecological cancer for which overall prognosis has remained poor over the past few decades. A number of theories have been postulated in an effort to explain the etiology of epithelial ovarian cancer, each of which has been both applauded and doubted. Of note, these theories likely are not mutually exclusive, as they all converge more or less on the role of inflammation in promoting ovarian tumorigenesis. In this review, we describe the latest studies on the role of inflammation in the initiation and progression of epithelian ovarian cancer from three major aspects: physiological functions of a normal ovary, potential involvement of the fallopian tube in the initiation of epithelian ovarian cancer and the strong impact of the cellular microenvironment on the development of the disease.

Introduction

Epithelial ovarian cancer (EOC), the most common subgroup of ovarian cancer, is the deadliest gynecological cancer in the United States, accounting for more deaths than all other gynecological cancers combined.[1] The high mortality rate for EOC is a result of technical obstacles to early detection of the disease and a high prevalence of distal metastasis at late stages of the disease [(70% of cases)[2]]. This latter property is probably attributable to the unique peritoneal environment of EOC, which facilitates convenient seeding of ovarian cancer cells in the peritoneal cavity, which is further aided by the constant flow of peritoneal fluid.[3] We call particular attention to this "open" environment to which EOC is exposed, because it has resulted in a myriad of characteristics specific to EOC, such as ease of widespread cancer metastases in a short period of time, unique formation of ascites, and high susceptibility of the ovarian surface epithelium (OSE) to peritoneal inflammatory stimuli.

*Correspondence to: Jinsong Liu; Email: jl u@mdanderson.org

Etiology of EOC: Inflammatory in Nature

EOC is perhaps one of the most sinuous human cancers. In an effort to identify the causes of EOC, a few hypotheses have been put forward. Two of these theories—the incessant ovulation hypothesis and the gonadotrophin hypothesis—are the most dominant in the ovarian cancer society. Proposed in the early 1970s, the incessant ovulation hypothesis attributes the formation of EOC to continuous damage and repair of the ovarian surface epithelia during cyclical ovulatory processes, which increase the chances for replicative DNA errors to be incorporated in ovarian epithelial cells.[4] The gonadotrophin hypothesis, on the other hand, suggests that excessive exposure of the ovarian surface epithelia to gonadotrophins can result in enhanced epithelial cell proliferation and malignant transformation.[5] A third theory emerged in the late 1990s which states that hormonal influences, including androgen and progesterone, have a major impact on the proliferation of the ovarian surface epithelia and, hence, EOC.[6]

Unlike that of the majority of other organs, the surface epithelium of the ovary is a natural continuant of the peritoneal lining and thus is directly exposed to any metabolic, environmental and xenobiotic stress present in the peritoneal cavity, most of which have inflammatory properties. However, the sources of inflammatory stimuli to which the ovary is exposed remain undercharacterized. In fact, more than a decade ago, the primary physiological function of the ovary, ovulation, was found to be pro-inflammatory in nature[7] and potentially mutagenic.[8] As we focus on the molecular events that take place in the pre-ovulatory ovary, we will find that as the pre-ovulatory follicle matures, the proximal ovarian epithelial cells proliferate[9] and then undergo apoptosis[10] to accommodate follicular growth. Meanwhile, the fibroblastic layers of the tunica albuginea and theca externa are weakened in preparation for ovulation. These events culminate in a burst of apical epithelial cells and the underlying follicular layers followed by rapid extrusion of the ovum, wounding the surface of the ovary.[11] Intriguingly, these ovulatory processes, together with the repair steps immediately after liberation of the ovum, are marked by generation of an enormous body of

cytokines/chemokines and matrix-remodeling enzymes, including prostaglandins, bioactive eicosanoids, plasminogen activators, collagenases, interleukins (ILs), tumor necrosis factors and various growth factors,[12,13] as well as by recruitment of activated immune cells to the wounded epithelial surface, implying the occurrence of global activation of the pro-inflammatory network. Thus, the strong inflammatory stimuli, being both triggers and natural by-products of ovulation, may cause additional damage to the ovarian surface epithelia, which is already under tremendous stress because of the ovulatory rupture of the local epithelial cell layer. Not surprisingly, this panel of inflammatory modulators activated during cyclical ovulation has been found to exhibit a striking overlap with that described for EOC, including IL-8, CCL2/MCP-1 and CCL5/RANTES.[14] Therefore, the incessant ovulation hypothesis has, perhaps inadvertently, provided evidence that inflammatory responses induced under physiological conditions may foster the development of EOC. Similarly, studies have shown that elevation of estrogens[15,16] and androgens,[17] as proposed by the gonadotrophin hypothesis and hormonal hypothesis, respectively, amplifies immune responses by recruiting pro-inflammatory cells and molecular effectors. Collectively, hypotheses attributing EOC to ovulation, gonadotrophin release, and hormonal influences likely are not mutually exclusive and lend strength to suggest that normal physiological activities of the ovary are accompanied by general activation of inflammatory mediators, which may either directly cause EOC or gradually produce genomic damage to the ovarian surface epithelia, until a future bolus dose of pathological stress brings the overall mutational tally above the threshold of ovarian tumorigenesis.

Inflammation, Tubal Tumorigenesis and Ovarian Cancer

Although the conventional view regarding ovarian cancer development is that more than 90% of cases originate from the OSE, the latest evidence points to hypothetical involvement of the fallopian tube, in particular, the fimbriated end of the tube, in the formation of serous ovarian cancer, prompted by findings presented by Crum et al. and other investigators. These authors demonstrated that examination of fallopian tubes and ovaries taken from BRCA-mutant women undergoing prophylactic salpingo-oophorectomy identified precursor lesions of serous ovarian carcinomas, unexpectedly, only in the tubal fimbria, not in the ovary.[18-20] Thus, at least in some cases, the fimbriated end of the fallopian tube may be the culprit in seeding of serous ovarian cancer.

Inflammatory insults to the fallopian tube can lead to tubal epithelial carcinogenesis. For instance, luminal dilatation and plical atrophy in the fallopian tube caused by chronic infection has been documented in many cases of primary fallopian tubal carcinomas.[21] Exposure of the fallopian tube to inflammatory insults may occur physiologically and pathologically. Under physiological conditions, the retrograde flow of endometrial fluid during menstruation renders the fallopian tube acutely inflammatory by exposing the tube to a plethora of inflammatory molecules, including IL-8, tumor necrosis factor-α, and granulocyte-macrophage

colony-stimulating factor, all of which have been shown to be elevated in ovarian tumor specimens.[22-24] Furthermore, the functional tubal fimbria, which has two epithelial surfaces—ciliated epithelium (endosalpinx) and peritoneal mesothelium—may be an area of continuous abrasion, stress-induced inflammation, and consequently, a site of cancer initiation.[25] Examples of endosalpinx-peritoneal junction-associated cancers include cervical[26] and gastroesophageal[27] malignancies, where the cervical squamous columnar junction and esophagogastric junction are located, respectively. In comparison, pathological inflammatory agents, including those that travel up from the lower female genital tract to the fallopian tube, are found frequently and to blame for a large proportion of female infertility. For example, pelvic exposure to asbestos[28] and to the sexually transmitted pathogen *Chlamydia trachomatis*[29] is known to cause tubal inflammation, also known as salpingitis. Taken together, these findings indicate that tubal inflammation is common under both pathological and non-pathological conditions.

Because inflammation is known to be a causal factor in promoting tubal tumorigenesis, the hypothesis that a portion of serous ovarian carcinomas may originate in the fallopian tube provides another link, although indirect, between inflammation and EOC. Recent studies, albeit preliminary, have associated inflammation of the fallopian tube with ovarian tumorigenesis, and supported studies indicating that the fallopian tube could be one of the origins of EOC. For example, the presence of chronic salpingitis has been found in 53% of ovarian carcinoma cases,[30] suggesting a causative relationship between the two. This notion is best supported by findings showing that hysterectomy and tubal ligation, both of which cut off the passage of inflammatory factors from the lower to the upper genital tract, afforded protection against EOC.[31] More importantly, hysterectomy alone without tubal ligation was less effective in protecting against EOC than was hysterectomy combined with tubal ligation,[32] emphasizing the significance of the fallopian tube in initiation of EOC. Although the hypothesis that (some of) serous ovarian cancers may stem from the tubal fimbria is still heatedly debated and calls for more substantial evidence, it for another time, perhaps unintentionally, supports the hypothesis that ovarian cancer is by nature inflammatory.

Inflammation, Cellular Senescence in the Ovarian Epithelial Microenvironment and Ovarian Cancer

As described above, the complex biology of the OSE makes ovarian epithelial cells exceedingly sensitive to peritoneal inflammatory agents. However, this is only half the story; the other half resides in the cellular microenvironment created by the ovarian stromal cells, in particular, aged or senescent stromal cells. Cellular senescence was initially described as an evolutionary advantage endowing cancer prevention when the cells entered an irreversible status of cell cycle arrest[33] in response to a variety of internal and external stimuli.[34,35] In contrast with the conventional view that senescence is inherently protective against cancer, mounting evidence points to an unexpected role of senescent stromal cells, mainly stromal fibroblasts, in enhancing epithelial
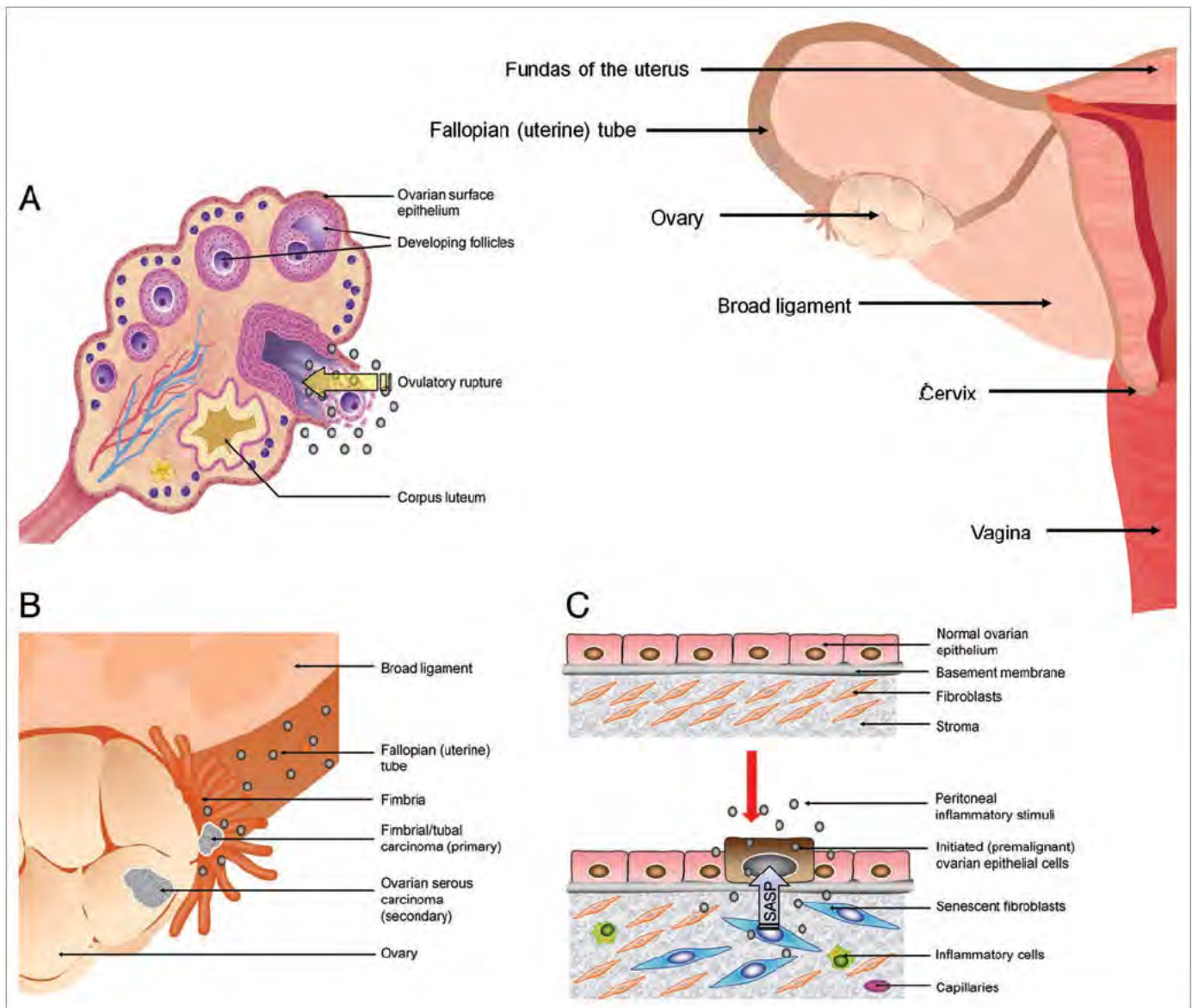
Figure 1. Potential sources of inflammatory stimuli that may contribute to the initiation and/or progression of EOC. A schematic representation of the left half of the female reproductive organs is shown at top right. (A) Normal functions of the ovary, such as ovulation, may be pro-inflammatory in nature. (B) Inflammatory insults to the fallopian tube can indirectly damage the adjacent ovarian surface epithelium. (C) Inflammatory molecules present in the peritoneal cavity may not only be mutagenic to the ovarian surface epithelium but also render ovarian stromal fibroblasts senescent. Subsequently, senescent fibroblasts create a secondary hyper-inflammatory microenvironment (SASP), together with inflammatory mediators in the peritoneal macroenvironment, contributing to the enhancement of EOC.

tumorigenesis. Specifically, Krtolica and colleagues showed that senescent but not normal human fibroblasts were markedly tumorigenic in premalignant (initiated but non-tumorigenic) human skin epithelial cells both in culture and in immune-compromised mice,[36] thus providing experimental proof that senescent stromal fibroblasts can augment epithelial tumorigenesis. Therefore, cellular senescence acts as a double-edged sword by either dampening or boosting tumorigenesis depending on the specific cell type and combination of intracellular and extracellular factors. Accumulating evidence has suggested that diffusible paracrine signaling molecules secreted by senescent fibroblasts orchestrate the senescence-associated enhancement of tumorigenesis by

fine-tuning the epithelial microenvironment into one favorable for tumor growth. Thus far, a broad spectrum of pro-inflammatory mediators have been reported to be markedly activated in senescent cells, including myriad ILs (e.g., IL-6, IL-8, IL-1β), chemoattractants (e.g., Gro-1/α, MCP-1, CSMF), matrix-remodeling enzymes (e.g., MMPs, TIMPs, uPAR), and adhesion molecules (e.g., ICAM-1, VCAM-1, integrins),[37] suggesting that upon senescence, aged cells take up the highly pro-inflammatory "senescence-associated secretory phenotype" (SASP).[38]

Our laboratory has found direct evidence that senescent ovarian fibroblasts promote ovarian epithelial tumorigenesis by mobilizing the pro-inflammatory network. Recently, we demonstrated

that expression of the chemokine Gro-1/α was induced in HRAS^{V12}-transformed ovarian epithelial cells and that epithelial cell-released Gro-1/α mediated the senescence of ovarian stromal fibroblasts by diffusing into the stroma and acting non-autonomously on fibroblasts.[39] Subsequently, ovarian fibroblasts rendered senescent by Gro-1/α proved to be tumor-promoting of initiated ovarian epithelial cells when co-injected into nude mice with the latter,[39] which was consistent with results reported previously by others. In addition to Gro-1/α, we have also observed elevated expression of a wide spectrum of pro-inflammatory cytokines and chemokines in HRAS^{V12}-transformed ovarian epithelial cells than in their immortalized, non-tumorigenic parental cells.[40] When this panel of RAS-induced secreted factors was compared with the SASP described in Coppe's study,[38] a considerable overlap between these two was identified, including IL-6, IL-8, Gro-1/α, Gro-2/β, ICAM-1, IGFBP-1 and MCP-1 (reviewed in refs. 40 and 41 and unpublished data from us). Some of these factors are established senescence inducers,[39,42-44] suggesting that many, if not all, of the HRAS^{V12}-induced inflammatory molecules could also mediate cellular senescence. Do senescent stromal fibroblasts enhance human EOC in vivo? The answer to this question is probably yes. We have detected senescent ovarian stromal fibroblasts adjacent to human ovarian tumor epithelium in clinical specimens,[39] supporting the existence of such cells in human cases of ovarian cancer. Although evidence supporting a senescence-associated pro-inflammatory secretome acting in a paracrine fashion on ovarian tumor epithelium in vivo has been lacking, postulating that inflammation-mediated stromal senescence can play a critical role in triggering as well as promoting human EOC is reasonable. Collectively, we have shown that in oncogenic RAS-transformed ovarian epithelial cells, a drastically pro-inflammatory secretome is generated, which can diffuse into the stroma and cause senescence in stromal fibroblasts. Conversely, senescence of ovarian stromal fibroblasts may contribute to progression of EOC by creating a secondary pro-inflammatory phenotype (SASP) and converting the ovarian epithelial microenvironment into one filled with inflammatory mediators that favor tumor advancement. The central role of the inflammatory network in interweaving these events is prominent, which directs extensive cellular communications between the ovarian tumor epithelium and the underlying stroma that converge on the augmentation of EOC.

Conclusions

The tumor milieu in which EOC develops has been described as one enriched with a broad spectrum of pro-inflammatory cytokines and chemokines.[14] Increasing evidence suggests that inflammation contributes significantly to the etiology of EOC. Studies have not only shown that physiological ovarian functions are pro-inflammatory in nature (**Fig. 1A**) but also suggested that activities that take place in the fallopian tubes influence EOC (**Fig. 1B**). More recent studies of cellular senescence have revealed a potential role for senescent stromal fibroblasts in the augmentation of EOC by increasing the expression of diffusible inflammatory mediators (**Fig. 1C**). Intriguingly, cellular senescence, which itself serves as a vast repertoire of inflammatory molecules, can be induced by physiologically and pathologically derived inflammatory agents in the peritoneal *macro*environment and/or cellular *micro*environment; when these stimuli work in synergy, the ovarian epithelial tumor milieu becomes exponentially inflammatory and favorable for cancer development. A more comprehensive understanding of these issues will benefit the cancer pharmaceutical industry in designing new strategies for the treatment and prevention of human EOC.

References

1. Jemal A, Murray T, Ward E, Samuels A, Tiwari RC, Ghafoor A, et al. Cancer statistics, 2005. CA Cancer J Clin 2005; 55:10-30.
2. Jemal A, Tiwari RC, Murray T, Ghafoor A, Samuels A, Ward E, et al. Cancer statistics, 2004. CA Cancer J Clin 2004; 54:8-29.
3. Naora H, Montell DJ. Ovarian cancer metastasis: integrating insights from disparate model organisms. Nat Rev Cancer 2005; 5:355-66.
4. Fathalla MF. Incessant ovulation—a factor in ovarian neoplasia? Lancet 1971; 2:163.
5. Cramer DW, Welch WR. Determinants of ovarian cancer risk II. Inferences regarding pathogenesis. J Natl Cancer Inst 1983; 71:717-21.
6. Risch HA. Hormonal etiology of epithelial ovarian cancer, with a hypothesis concerning the role of androgens and progesterone. J Natl Cancer Inst 1998; 90:1774-86.
7. Bonello N, McKie K, Jasper M, Andrew L, Ross N, Braybon E, et al. Inhibition of nitric oxide: effects on interleukin-1beta-enhanced ovulation rate, steroid hormones, and ovarian leukocyte distribution at ovulation in the rat. Biol Reprod 1996; 54:436-45.
8. Ness RB, Cottreau C. Possible role of ovarian epithelial inflammation in ovarian cancer. J Natl Cancer Inst 1999; 91:1459-67.
9. Murdoch WJ. Ovarian surface epithelium during ovulatory and anovulatory ovine estrous cycles. Anat Rec 1994; 240:322-6.
10. Murdoch WJ. Programmed cell death in preovulatory ovine follicles. Biol Reprod 1995; 53:8-12.
11. Murdoch WJ. Ovarian surface epithelium, ovulation and carcinogenesis. Biol Rev Camb Philos Soc 1996; 71:529-43.
12. Espey L. Ovulation as an inflammatory process. In: Sjoberg N, Hamberger L, Janson PO, Owman CH, Coelingh-Bennink HJT, ed. Local Regulation of Ovarian Function. Park Ridge: Parthenon Publishing Group 1992; 183-200.
13. Espey L, Lipner H. Ovulation. In: Knobil E, Neill JD, ed. The Physiology of Reproduction. New York: Raven Press 1994.
14. Freedman RS, Deavers M, Liu J, Wang E. Peritoneal inflammation—A microenvironment for epithelial ovarian cancer (EOC). J Transl Med 2004; 2:23.
15. Crowley-Nowick P. Basic principles of immunology and genital tract immunity. In: Ness R, Kuller LH, ed. Health and disease among women. Biological and environmental influences. New York: Oxford University Press 1998; 273-96.
16. Manzi S, Ramsey-Goldman R. Autoimmune diseases. In: Ness R, Kuller LH, ed. Health and disease among women. Biological and environmental influences. New York: Oxford University Press 1998; 342-72.
17. Orvieto R, Fisch N, Yulzari-Roll V, La Marca A. Ovarian androgens but not estrogens correlate with the degree of systemic inflammation observed during controlled ovarian hyperstimulation. Gynecol Endocrinol 2005; 21:170-3.
18. Crum CP, Drapkin R, Kindelberger D, Medeiros F, Miron A, Lee Y. Lessons from BRCA: the tubal fimbria emerges as an origin for pelvic serous cancer. Clin Med Res 2007; 5:35-44.
19. Medeiros F, Muto MG, Lee Y, Elvin JA, Callahan MJ, Feltmate C, et al. The tubal fimbria is a preferred site for early adenocarcinoma in women with familial ovarian cancer syndrome. Am J Surg Pathol 2006; 30:230-6.

20. Callahan MJ, Crum CP, Medeiros F, Kindelberger DW, Elvin JA, Garber JE, et al. Primary fallopian tube malignancies in BRCA-positive women undergoing surgery for ovarian cancer risk reduction. J Clin Oncol 2007; 25:3985-90.

21. Demopoulos RI, Aronov R, Mesia A. Clues to the pathogenesis of fallopian tube carcinoma: a morphological and immunohistochemical case control study. Int J Gynecol Pathol 2001; 20:128-32.

22. Maisey K, Nardocci G, Imarai M, Cardenas H, Rios M, Croxatto HB, et al. Expression of proinflammatory cytokines and receptors by human fallopian tubes in organ culture following challenge with *Neisseria gonorrhoeae*. Infect Immun 2003; 71:527-32.

23. McGee ZA, Jensen RL, Clemens CM, Taylor-Robinson D, Johnson AP, Gregg CR. Gonococcal infection of human fallopian tube mucosa in organ culture: relationship of mucosal tissue TNFalpha concentration to sloughing of ciliated cells. Sex Transm Dis 1999; 26:160-5.

24. Strandell A, Thorburn J, Wallin A. The presence of cytokines and growth factors in hydrosalpingeal fluid. J Assist Reprod Genet 2004; 21:241-7.

25. Carvalho JP, Carvalho FM. Is Chlamydia-infected tubal fimbria the origin of ovarian cancer? Med Hypotheses 2008; 71:690-3.

26. Schiffman M, Castle PE, Jeronimo J, Rodriguez AC, Wacholder S. Human papillomavirus and cervical cancer. Lancet 2007; 370:890-907.

27. Nunobe S, Nakanishi Y, Taniguchi H, Sasako M, Sano T, Kato H, et al. Two distinct pathways of tumorigenesis of adenocarcinomas of the esophagogastric junction, related or unrelated to intestinal metaplasia. Pathol Int 2007; 57:315-21.

28. Heller DS, Gordon RE, Katz N. Correlation of asbestos fiber burdens in fallopian tubes and ovarian tissue. Am J Obstet Gynecol 1999; 181:346-7.

29. Cooper MD, Rapp J, Jeffery-Wiseman C, Barnes RC, Stephens DS. *Chlamydia trachomatis* infection of human fallopian tube organ cultures. J Gen Microbiol 1990; 136:1109-15.

30. Seidman JD, Sherman ME, Bell KA, Katabuchi H, O'Leary TJ, Kurman RJ. Salpingitis, salpingoliths and serous tumors of the ovaries: is there a connection? Int J Gynecol Pathol 2002; 21:101-7.

31. Green A, Purdie D, Bain C, Siskind V, Russell P, Quinn M, et al. Tubal sterilisation, hysterectomy and decreased risk of ovarian cancer. Survey of Women's Health Study Group. Int J Cancer 1997; 71:948-51.

32. Ness RB, Grisso JA, Cottreau C, Klapper J, Vergona R, Wheeler JE, et al. Factors related to inflammation of the ovarian epithelium and risk of ovarian cancer. Epidemiology 2000; 11:111-7.

33. Hayflick L, Moorhead PS. The serial cultivation of human diploid cell strains. Exp Cell Res 1961; 25:585-621.

34. Parrinello S, Coppe JP, Krtolica A, Campisi J. Stromal-epithelial interactions in aging and cancer: senescent fibroblasts alter epithelial cell differentiation. J Cell Sci 2005; 118:485-96.

35. Campisi J, d'Adda di Fagagna F. Cellular senescence: when bad things happen to good cells. Nat Rev Mol Cell Biol 2007; 8:729-40.

36. Krtolica A, Parrinello S, Lockett S, Desprez PY, Campisi J. Senescent fibroblasts promote epithelial cell growth and tumorigenesis: a link between cancer and aging. Proc Natl Acad Sci USA 2001; 98:12072-7.

37. Shan W, Yang G, Liu J. The inflammatory network: bridging senescent stroma and epithelial tumorigenesis. Front Biosci 2009; 14:4044-57.

38. Coppe JP, Patil CK, Rodier F, Sun Y, Munoz DP, Goldstein J, et al. Senescence-associated secretory phenotypes reveal cell-nonautonomous functions of oncogenic RAS and the p53 tumor suppressor. PLoS Biol 2008; 6:2853-68.

39. Yang G, Rosen DG, Zhang Z, Bast RC Jr, Mills GB, Colacino JA, et al. The chemokine growth-regulated oncogene 1 (Gro-1) links RAS signaling to the senescence of stromal fibroblasts and ovarian tumorigenesis. Proc Natl Acad Sci USA 2006; 103:16472-7.

40. Liu J, Yang G, Thompson-Lanza JA, Glassman A, Hayes K, Patterson A, et al. A genetically defined model for human ovarian cancer. Cancer Res 2004; 64:1655-63.

41. Coppe JP, Kauser K, Campisi J, Beausejour CM. Secretion of vascular endothelial growth factor by primary human fibroblasts at senescence. J Biol Chem 2006; 281:29568-74.

42. Acosta JC, O'Loghlen A, Banito A, Guijarro MV, Augert A, Raguz S, et al. Chemokine signaling via the CXCR2 receptor reinforces senescence. Cell 2008; 133:1006-18.

43. Kuilman T, Michaloglou C, Vredeveld LC, Douma S, van Doorn R, Desmet CJ, et al. Oncogene-induced senescence relayed by an interleukin-dependent inflammatory network. Cell 2008; 133:1019-31.

44. Wajapeyee N, Serra RW, Zhu X, Mahalingam M, Green MR. Oncogenic BRAF induces senescence and apoptosis through pathways mediated by the secreted protein IGFBP7. Cell 2008; 132:363-74.

Exhibit 81

New Insights into the Pathogenesis of Ovarian Cancer: Oxidative Stress

Ghassan M. Saed, Robert T. Morris and
Nicole M. Fletcher

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.73860

Abstract

Ovarian cancer is the leading cause of death from gynecologic malignancies yet the underlying pathophysiology is not clearly established. Epithelial ovarian cancer (EOC) has long been considered a heterogeneous disease with respect to histopathology, molecular biology, and clinical outcome. Treatment of ovarian cancer includes a combination of cytoreductive surgery and combination chemotherapy, with platinums and taxanes. Despite initial success, over 75% of patients with advanced disease will relapse around 18 months and the overall 5-year survival is approximately 50%. Cancer cells are known to be under intrinsic oxidative stress, which alters their metabolic activity and reduces apoptosis. Epithelial ovarian cancer has been shown to manifest a persistent pro-oxidant state as evident by the upregulation of several key oxidant enzymes in EOC tissues and cells. In the light of our scientific research and the most recent experimental and clinical observations, this chapter provides the reader with up to date most relevant findings on the role of oxidative stress in the pathogenesis and prognosis of ovarian cancer, as well as a novel mechanism of apoptosis/survival in EOC cells.

Keywords: ovarian cancer, oxidative stress, chemoresistance, apoptosis, nitrosylation, caspase-3

1. Introduction

Ovarian cancer is the fifth leading cause of cancer death; the leading cause of death from gynecologic malignancies, and the second most commonly diagnosed gynecologic malignancy; yet the underlying pathophysiology continues to be delineated [1, 2]. Epithelial ovarian cancer

has long been considered a heterogeneous disease with respect to histopathology, molecular biology, and clinical outcome. It comprises at least five distinct histological subtypes, the most common and well-studied being high-grade serous ovarian cancer (HGSOC) [3]. The majority of advanced-stage tumors are of epithelial cell origin and can arise from serous, mucinous, or endometrioid cells on the surface epithelium of the ovary or the fallopian tube [2]. The most obvious clinical implication of tumor heterogeneity is that molecular-targeted therapy, while being effective at one tumor site, may not be as effective at all of them [3].

Because early-stage ovarian cancer presents with nonspecific symptoms, most often diagnosis is not made until after the malignancy has spread beyond the ovaries [4]. Mortality rates for this type of malignancy are high because of a lack of a sensitive and specific early-stage screening method [4]. Surgical cytoreduction followed by platinum/taxane chemotherapy results in complete clinical response in 50–80% of patients with stage III and IV disease, but most will relapse within 18 months and ultimately develop chemoresistant disease [2]. Resistance to chemotherapy can either be intrinsic, occurring at the onset of treatment, or acquired, when the disease recurs despite an initially successful response [5–7]. Attempts to overcome drug resistance are central to both clinical and basic molecular research in cancer chemotherapy [5, 8]. Cancer cells are known to be under intrinsic oxidative stress, resulting in increased DNA mutations or damage, genome instability, and cellular proliferation [9–13]. The persistent generation of cellular reactive oxygen species (ROS) is a consequence of many factors including exposure to carcinogens, infection, inflammation, environmental toxicants, nutrients, and mitochondrial respiration [14–17].

The origin and causes of ovarian tumors remains under debate. Injury to surface epithelial ovarian cells due to repeated ovulation is thought to induce tumorigenesis in these cells and is known as the "incessant ovulation hypothesis." Additionally, hormonal stimulation of the surface epithelium of the ovary has been described to initiate tumorigenesis in surface epithelial cells and is known as the "gonadotropin hypothesis." Moreover, the fallopian tube, and not the ovary, has been suggested to be the origin for most epithelial ovarian cancer [2, 18, 19]. Nevertheless, many cases of ovarian cancer continue to be described as *de novo*.

Histopathologic, clinical and molecular genetic profiles were successfully utilized to clearly discriminate between type I and type II ovarian tumors [19]. Accordingly, type I ovarian tumors develop from benign precursor lesions that implant on the ovary and include clear cell, endometrioid, low-grade serous carcinomas, mucinous carcinomas and malignant Brenner tumors [19]. Type II ovarian tumors develop from intraepithelial carcinomas of the fallopian tube and can then spread to involve both the ovary as well as other sites, such as high-grade serous carcinomas which comprise morphologic and molecular subtypes [19]. Additionally, high-grade endometrioid, poorly differentiated ovarian cancers, and carcinosarcomas are also classified as type II tumors.

Attempts to identify specific genes in ovarian tumors to help in early detection of the disease and serve as targets for improved therapy had failed to identify reproducible prognostic indicators [2, 20–22]. Several oncogenic mutations and pathways have been identified in ovarian cancer. Specific inherited mutations in the *BRCA1* and *BRCA2* genes that produce tumor suppressor proteins, are known to be associated with a 15% increased risk of ovarian cancer overall [2]. Ovarian cancers associated with *BRCA1* and *BRCA2* mutations are much more common in

younger age patients as compared with their nonhereditary counterparts. Additionally, somatic gene mutations in RAD51C and D, HNPCC, NF1, RB1, CDK12, P53, BRAF, KRAS, PIK3CA, and PTEN have been identified in epithelial ovarian cancer. Somatic mutations in BRAF and KRAS genes are relatively common in type I tumors, while p53 mutations, RAS signaling and PIK3CA are common in type II. Additional genetic variations have been hypothesized to act as low to moderate alleles, which contribute to ovarian cancer risk, as well as other diseases [23].

Ovarian tumors are distinct from many other type of cancers as they rarely metastasize outside of the peritoneal cavity [24]. Ovarian tumors are spread into the peritoneal cavity when cells from the primary tumor detach and travel into the peritoneum where they implant into the mesothelial lining [25]. Metastases beyond the peritoneum are usually restricted to recurrent or advanced disease; however, pleural metastases were reported to be present at initial diagnosis. Moreover, the recent discovery of ovarian cancer stem cells, which manifest properties of typical cancer stem cells, in ascites is a new additional contributing factor to not only to metastasis but also to chemoresistance [25, 26].

2. Oxidative stress

Homeostasis, the balance between the production and elimination of oxidants, is maintained by mechanisms involving oxidants and antioxidant enzymes and molecules. If this balance is altered, it leads to an enhanced state of oxidative stress that alters key biomolecules and cells of living organism [13]. Oxidant molecules are divided into two main groups; oxygen-derived or nitrogen-containing molecules. Oxygen-derived molecules, also known as reactive oxygen species (ROS), includes free radicals such as hydroxyl ($HO^{\bullet}$), superoxide ($O_2^{\bullet-}$), peroxyl ($RO_2^{\bullet}$), and alkoxyl ($RO^{\bullet}$), as well as oxidizing agents such as hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), ozone (O_3), and singlet oxygen ($^1O_2$) that can be converted to radicals [13, 27]. Nitrogen containing oxidants, also known as reactive nitrogen species (RNS), are derived from nitric oxide (NO) that is produced in the mitochondria in response to hypoxia [13]. Exposure to inflammation, infection, carcinogens, and toxicants are major sources of ROS and RNS, *in vivo* [13, 16, 27, 28]. Additionally, RNS and ROS can be produced by various enzymes including cytochrome P450, lipoxygenase, cyclooxygenase, nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase complex, xanthine oxidase (XO), and peroxisomes (**Figure 1**) [13, 28, 29].

To maintain the redox balance, ROS and RNS are neutralized by various important enzyme systems including superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST), glutathione (GSH), thioredoxin coupled with thioredoxin reductase, glutaredoxin, glutathione peroxidase (GPX), and glutathione reductase (GSR) (**Figure 1**) [27]. Superoxide dismutase is known to convert $O_2^{\bullet-}$ to H_2O_2, which is then converted to water by CAT. Glutathione S-transferase is involved in detoxification of carcinogens and xenobiotics by catalyzing their conjugation to GSH that will aid in expulsion from the cell (**Figure 1**) [27]. Indeed, the GSH-to-oxidized-GSH (GSH/GSSG) ratio is a good indicator of cellular redox buffering capacity [30, 31]. Under enhanced oxidative stress, the GSH/GSSG complex is known to stimulate the activity of the GS-X-MRP1 efflux pump, which removes toxins from cells. This mechanism has been investigated in the development of resistance to chemotherapeutic drugs [30, 31].
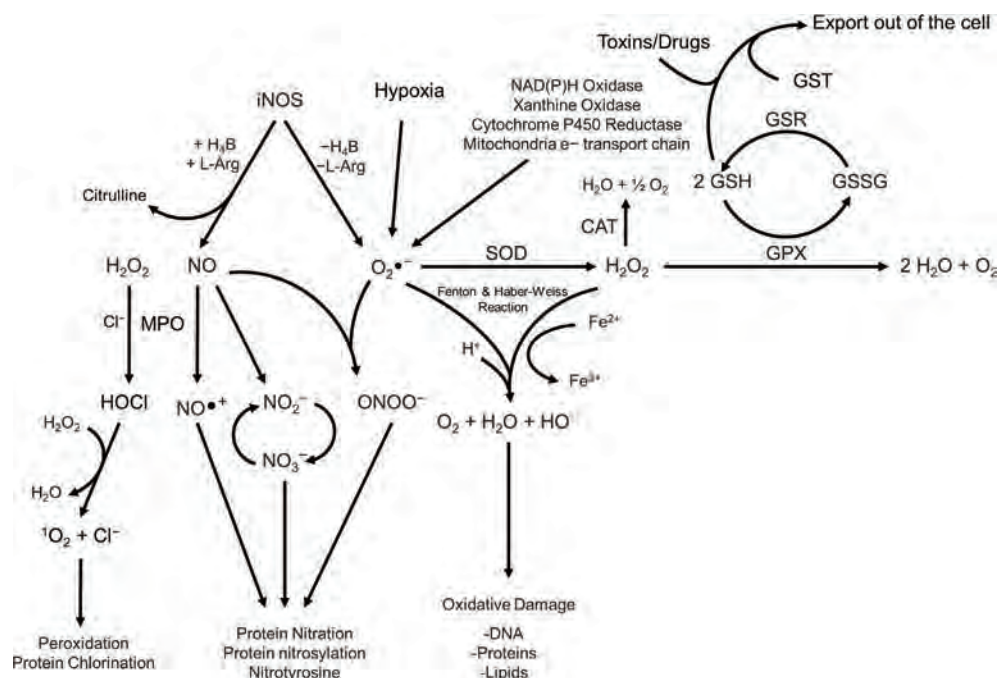
Figure 1. Summary of key oxidant and antioxidants in cancer [1]. Abbreviations are CAT, catalase; Cl⁻, chloride ion; Fe$_2$, iron (II); Fe$_3$, iron (III); GPX, glutathione peroxidase; GSH, glutathione; GSR, glutathione reductase; GSSG, reduced glutathione; GST, glutathione S-transferase; H_2O_2, hydrogen peroxide; H_4B, tetrohydrobiopterin; HO•, hydroxyl radical; HOCl, hypochlorous acid; iNOS, inducible nitric oxide synthase; L-Arg, L-arginine; MPO, myeloperoxidase; NAD(P)H, nicotinamide adenine dinucleotide phosphate; NO• , nitrosonium cation; NO_2^-, nitrite; NO_3^-, nitrate; $O_2^{•-}$, superoxide; ONOO⁻, peroxynitrite; SOD, superoxide dismutase.

3. Oxidative stress and cancer

Oxidative stress has been implicated in the etiology of several diseases, including cancer. Alteration of the cellular redox balance modulates the initiation, promotion, and progression of tumor cells [13, 27]. The continuous generation of oxidants and free radicals affects key cellular mechanisms that control the balance of cell proliferation and apoptosis, which play a major role in the initiation and development of several cancers. Depending on the concentration of ROS and RNS in the cellular environment, oxidants can initiate and promote the oncogenic phenotype or induce apoptosis, and thus act as antitumor agents [32]. Several transcription factors that modulate the expression of genes critical to the development and metastasis of cancer cells are known to be controlled by oxidative stress. This includes hypoxia inducible factor (HIF)-1α, nuclear factor (NF)-κB, peroxisome proliferator-activated receptor (PPAR)-γ, activator protein (AP)-1, β-catenin/Wnt, and Nuclear factor erythroid 2-related factor 2 (Nrf2) [13]. The transcription factor regulator Nrf2 is known to control the expression of some key antioxidant enzymes that are needed to scavenge oxidants and free radicals [13, 33]. The activation of Nrf2 involves the suppressor protein, Kelch-like ECH-associated protein 1

(Keap1), that binds Nrf2 in the cytoplasm and prevents its translocation into the nucleus, where it binds to promoters of antioxidant enzymes [13, 33]. Additionally, oxidative stress is known to activate certain signaling pathways, specifically, the MAPK/AP-1 and NF-κB pathways, which are critical for the initiation and maintenance of the oncogenic phenotype [34].

More importantly, ROS and RNS are known to induce genetic mutations that alter gene expression as well as induce DNA damage, and thus have been implicated in the etiology of several diseases, including cancer [2, 13, 35]. Damage to DNA by ROS and RNS is now accepted as a major cause of cancer, and has been demonstrated in the initiation and progression of several cancers including breast, hepatocellular carcinoma, and prostate cancer [34]. Oxidative stress is known to modify all the four DNA bases by base pair substitutions rather than base deletions and insertions. Modification of GC base pairs usually results in mutations, whereas, modification of AT base pairs does not [36]. Modification of guanine in cellular DNA, causing G to T transversions, is commonly induced by ROS and RNS [34]. If not repaired, the transversion of G to T in the DNA of oncogenes or tumor suppressor genes can lead to initiation and progression of cancer. Oxidation of DNA bases, such as thymidine glycol, 5-hydroxymethyl-2′-deoxyuridine, and 8-OHdG are now accepted markers of cellular DNA damage by free radicals [35].

Oxidants and free radicals are known to enhance cell migration contributing to the enhancement of tumor invasion and metastasis, main causes of death in cancer patients [2, 13]. Reactive oxygen species, through the activation of NF-κB, regulate the expression of intercellular adhesion protein-1 (ICAM-1), a cell surface protein in various cell types [13]. In response to oxidative stress, the interleukin 8 (IL-8)-induced enhanced expression of ICAM-1 on neutrophils enhances the migration of neutrophils across the endothelium, which is key in tumor metastasis [13]. Another important player that controls cell migration and consequently, tumor invasion, is the upregulation of specific matrix metalloproteinases (MMPs), essential enzymes in the degradation of most components of the basement membrane and extracellular matrix, such as type IV collagen [13, 37]. The expression of MMPs, such as MMP-2, MMP-3, MMP-9, MMP-10, and MMP-13 is enhanced by free radicals, specifically H_2O_2 and NO, through the activation of Ras, ERK1/2, p38, and JNK, or the inactivation of phosphatases [13, 37]. Indeed, the major source of cellular ROS, the NAD(P)H oxidase family of enzymes, has been linked to the promotion of survival and growth of tumor cells in pancreatic and lung cancers [2, 13].

Oxidants and free radicals are also known to enhance angiogenesis, a key process for the survival of solid tumors [13]. Angiogenesis involves the upregulation of vascular endothelial growth factor (VEGF) or the downregulation of thrombospondin-1 (TSP-1), an angiogenesis suppressor in response to oxidative stress [13]. This process is controlled by several oncogenes and tumor-suppressor genes such as Ras, c-Myc, c-Jun, mutated p53, human epidermal growth factor receptor-2, and steroid receptor coactivators [38, 39]. Additionally, oxidants and free radicals are known to stabilize HIF-1α protein and induce the production of angiogenic factors by tumor cells.

4. Cancer cells are under intrinsic oxidative stress

Cancer cells are continuously exposed to high levels of intrinsic oxidative stress due to increased aerobic glycolysis (Warburg effect), a known process in cancer cell metabolism [10, 40].

Thus, cancer cells trigger several critical adaptations that are essential for their survival such as suppression of apoptosis, alteration of glucose metabolism, and stimulation of angiogenesis [10, 29]. Oxygen depletion, due to a hypoxic microenvironment, significantly stimulates mitochondria to produce high levels of ROS and RNS which is known to activate HIF-1α and consequently promote cell survival in such an environment [29]. The half-life of HIF-1α is extremely short as it is rapidly inactivated through hydroxylation reactions mediated by dioxygen, oxaloglutarate, and iron-dependent prolyl 4-hydroxylases, located in the nucleus and cytoplasm [40, 41]. Nitric oxide and other ROS, as well as H_2O_2 efflux into the cytosol due to dismutation of $O_2^{\bullet-}$, can inhibit prolyl 4-hydroxylases activity, leading to the stabilization of HIF-1α [29, 42]. More importantly, stabilization of HIF-1α, under hypoxic conditions, can be blocked when inhibiting ROS production in mitochondria that lack cytochrome c [29, 43].

Pro-oxidant enzymes such as myeloperoxidase (MPO), inducible nitric oxide synthase (iNOS) and NAD(P)H oxidase have been associated with initiation, progression, survival, and increased risk in cancers such as breast, ovarian, lung, prostate, bladder, colorectal and malignant melanoma [21, 44]. Moreover, the expression of those key pro-oxidant enzymes was found to change based on the histological type and grade of the tumor [21, 45, 46]. Likewise, antioxidants have also been associated with initiation, progression, survival, and increased risk in cancers such as lung, head and neck, and prostate cancer [47–50]. The expression of GSR and GPX, key antioxidant enzymes, has also been reported to be altered in various types of cancer [21]. The activity and expression of SOD, a powerful antioxidant enzyme, has been reported to be decreased in colorectal carcinomas, pancreatic, lung, gastric, ovarian, and breast cancers [21, 45, 46]. Likewise, the expression and activity of CAT, a key antioxidant enzyme, was reported to be decreased in breast, bladder, and lung cancers but increased in brain cancer [21, 45, 46]. Antioxidant enzymes play a critical role in maintaining the redox balance in the presence of microenvironment stress, and thus, alteration of this balance may provide a unique and complex microenvironment for cancer cell survival.

5. Ovarian cancer cells manifest a persistent pro-oxidant state

Recent evidence suggests that oxidative stress is a critical factor in the initiation and development of several cancers, including ovarian cancer [40, 51]. Consistently, it has been reported that ovarian cancer patients manifested significantly decreased levels of antioxidants and higher levels of oxidants [10, 22, 40, 51–53]. An enhanced redox state, resulting from increased expression of key pro-oxidant enzymes and decreased expression of antioxidant enzymes, has been extensively described in epithelial ovarian cancer (EOC) [52–54]. We have previously reported that MPO, a hemoprotein present solely in myloid cells that acts as a powerful oxidant, and iNOS, a key pro-oxidant enzyme, are highly expressed and co-localized to the same cell in EOC cells [53]. These two enzymes, MPO and iNOS, work together to inhibit apoptosis, a hallmark of ovarian cancer cells. Nitric oxide, produced by iNOS, is used by MPO as a one-electron substrate to generate nitrosonium cation (NO$^+$), a labile nitrosating species, resulting in a significant increase in S-nitrosylation of caspase-3, which inhibits apoptosis [53, 55, 56]. Indeed, attenuating oxidative stress by inhibiting MPO or iNOS significantly induced

apoptosis in EOC cells [54]. Moreover, the remarkably higher levels of iNOS/NO, produced by EOC cells, resulted in the generation of high levels of nitrate and nitrite, powerful protein nitration agents that are known to stimulate the initiation and progression of tumor cells [53]. Under oxidative stress, where both NO and $O_2^{\bullet-}$ are elevated, MPO was reported to serve as a source of free iron which reacts with H_2O_2 and generated highly reactive hydroxyl radical ($HO^\bullet$), further increasing oxidative stress [22, 53]. Additionally, EOC cells are also character-ized by enhanced expression of NAD(P)H oxidase, a potent oxidant enzyme that is known to be the major source of $O_2^{\bullet-}$ in the cell. Such high levels of $O_2^{\bullet-}$ combined with significantly high levels of NO generates peroxynitrite, another powerful nitrosylation and nitration agent, which modifies proteins and DNA structure and function in cells [57].

Recently we have gathered compelling evidence demonstrating that talc, through alteration of the redox balance, can generate a similar pro-oxidant state in both normal ovarian epithe-lial and ovarian cancer cells. Talc and asbestos are both silicate minerals, and the carcinogenic effects of asbestos have been extensively studied and documented in the medical literature [58]. Asbestos fibers in the lung initiate an inflammatory and scarring process, and it has been proposed that ground talc, as a foreign body, might initiate a similar inflammatory response [58]. Although there is strong epidemiological evidence to suggest an association between talc use and ovarian cancer, the direct link and precise mechanisms have yet to be elucidated. We investigated the effect of talc on both oxidants and antioxidants in normal ovarian epithelial and ovarian cancer cell lines. There was a marked increase in mRNA levels of the pro-oxidant enzymes, iNOS and MPO in talc treated ovarian cancer cell lines and normal ovarian epi-thelial cells, all as compared to their control, as early as 24 hours. Additionally, there was a marked decrease in the mRNA levels of the antioxidant enzymes CAT, GPX, SOD3, but with a marked increase in GSR, and no change in GST, in talc treated ovarian cancer cell line and in normal ovarian epithelial cells, all compared to their control, as early as 24 hours (*data not pub-lished*). Thus, there is a direct effect of talc on the molecular levels of oxidant and antioxidants, elucidating a potential mechanism for the development of ovarian cancer in response to talc.

6. Biomarkers for the early detection of ovarian cancer

The discovery of MPO expression in ovarian EOC cells and tissues was surprising, as it is only expressed by cells of myeloid origin. Intriguingly, the combination of serum MPO and free iron was reported to potentially serve as biomarkers for early detection of ovarian cancer [22]. A robust detection method based on molecular profiles for ovarian cancer has not yet been developed because the disease exhibits a wide range of morphological, clinical and genetic variations during its progression. The search for non-invasive, cost-effective ovarian cancer biomarker tests has been ongoing for many years. Immunizations of mice with ovarian cancer cells has led to hybridoma validation by ELISA, while flow cytometry analysis permitted the discovery of cancer antigen (CA)-125 and mesothelin [59]. Furthermore, the screening of an array of 21,500 unknown ovarian cDNAs hybridized with labeled first-strand cDNA from ten ovarian tumors and six normal tissues led to the discovery of human epididymis protein 4 (HE4) [60]. Most interestingly, HE4 is overexpressed in 93% of serous and 100% of endometrioid

EOCs, and in 50% of clear cell carcinomas, but not in mucinous ovarian carcinomas [61]. Thus, HE4 was identified as one of the most useful biomarkers for ovarian cancer, although it lacked tissue-specificity [60, 62–64]. Secreted HE4 high levels were also detected in the serum of ovarian cancer patients [65]. Additionally, combining CA-125 and HE4 is a more accurate predictor of malignancy than either alone [66–68].

Multi-marker panels have the potential for high positive predictive values (PPVs), but careful validation with appropriate sample cohorts is mandatory and complex algorithms may be difficult to implement for routine clinical use [59]. Panels of biomarkers have been extensively investigated to improve sensitivity and specificity and have included some of the most promising reported markers such as CA72–4, M-CSF, OVX1, LPA, prostacin, osteopontin, inhibin and kallikrein [69–71]. However, most of these tests frequently require certain equipments and complex computational algorithms that may not be available in a standard immunoassay laboratory, [32]. Among postmenopausal women in the U.S., only 1 in 2500 women are reported with ovarian cancer. Due to this low prevalence of the disease, a screening method that yield a 75% sensitivity and 99.6% specificity to achieve a PPV value of 10% to be effective for the detection of all stages of ovarian cancer [72]. To date, there is no single biomarker available that met these requirements.

The established role of MPO in oxidative stress and inflammation has been a leading factor in the study of MPO as a possible marker of plaque instability and a useful clinical tool in the evaluation of patients with coronary heart disease [73]. Recent genetic studies implicated MPO in the development of lung cancer by demonstrating a striking correlation between the relative risk for development of the disease and the incidence of functionally distinct MPO polymorphisms [74]. Myeloperoxidase levels reported for various inflammatory disorders are coincidentally lower than those levels found in all stages of ovarian cancer. A previous study reported normal serum MPO and iron levels as 62 ± 11 ng/ml and 96 ± 9 µg/dl, respectively [75]. However, there was a significant increase in serum MPO and iron levels to 95 ± 20 ng/ml and 159 ± 20 µg/dl, respectively, in asthmatic individuals [75]. Although there was an increase in this reported serum iron, these levels still fell within the normal range (50 to 170 µg/dl) [22, 75]. Other studies have showed that an elevated MPO levels, reaching up to 350 ng/ml, in serum plasma, was indicative of a higher risk for cardiovascular events in patients hospitalized for chest pain [76, 77]. A recent study showed a significant correlation between MPO levels and the stage of ovarian cancer, as is the linear trend for MPO with increasing stage [22]. Similarly, there was a significant difference in the level of free iron in serum and tissues obtained from stage I as compared to combined stages II, III, and IV ovarian cancer. There was an overlap between stage I ovarian cancer and inflammation (endometriosis) serum MPO levels, however serum free iron levels were significantly higher in stage I ovarian cancer as compared to inflammation. There was no significant change in free iron levels between the healthy control group, benign gynecologic conditions group, and inflammation group [22].

Due to the overlap of MPO levels in early-stage ovarian cancer and inflammatory conditions, there is a potential for a false positive with MPO alone in patients with cardiovascular, inflammation, and/or asthmatic disorders. It has been reported that MPO heme destruction and iron release is mediated by high levels of both HOCl (a product of MPO) and oxidative stress (i.e. cancer) [22]. The free iron generated by hemoprotein destruction not only contributes to elevation of

serum iron levels, but may also induce oxidative stress, which can promote lipid peroxidation, DNA strand breaks, and modification or degradation of biomolecules [78–80]. Iron reacts with H_2O_2 and catalyzes the generation of highly reactive hydroxyl radicals, which in turn further increases free iron concentrations by the Fenton and Haber–Weiss reaction [81]. Several studies from our laboratories have provided a mechanistic link between oxidative stress, MPO, higher levels of HOCl and higher free iron that could explain the observed accumulation of free iron in epithelial ovarian cancers tissues [53, 82–85]. Utilizing serum iron levels alone as a biomarker is also not sufficient for early detection of ovarian cancer due to many uncontrolled variables, i.e. dietary intake, supplements, effects of other iron-generating enzymes or factors, and more importantly they are not as specific as MPO levels. Specifically, in iron deficiency anemic patients, their free iron levels may become a confounding factor in its utilization for early detection of ovarian cancer. Thus, anemia should be ruled out to eliminate any overlap that would lead to misdiagnosis. The incorporation of iron deficiency anemic patients in a logistic regression model will help determine its overlap with early-stage ovarian cancer. Additionally, currently available clinical studies focused on either biochemical or more recently, genetic markers of iron overload have reported conflicting results regarding the use of iron levels alone for diagnosis [86–89].

Thus, the combination of serum MPO and iron levels should yield a higher power of specificity and sensitivity that should distinguish women with early-stage ovarian cancer from other disorders, specifically inflammation [22]. Additionally, combining serum MPO and iron levels with the best currently existing biomarkers through the creation of a logistic regression model may increase the overall predictive values. Collectively, there is a role for serum MPO and free iron in the pathophysiology of ovarian cancer, which thereby qualifies them to serve as biomarkers for early detection and prognosis of ovarian cancer.

7. Modulation of oxidative stress

Several studies have reported the beneficial effects of modulating the redox status of cancer cells, however few studies have been reported for ovarian cancer [90–92]. Inhibition of pro-oxidant enzymes, such as NAD(P)H oxidase, has been shown to significantly induce apoptosis of cancer cells [93, 94]. We investigated whether NAD(P)H oxidase-mediated generation of intracellular reactive ROS lead to anti-apoptotic activity and thus a growth advantage to EOC cells. Diphenyleneiodonium (DPI) has been used to inhibit ROS production mediated by NAD(P)H oxidase in various cell types [95–97]. Our results showed that NAD(P)H oxidase is over-expressed in EOC tissues and cells as compared to normal ovarian tissues and cells [52]. Indeed, high levels of NAD(P)H oxidase are known to promote tumorigenesis of NIH3T3 mouse fibroblasts and the DU-145 prostate epithelial cells [98].

Inhibition of NAD(P)H oxidase has also been reported to decrease the generation of $O_2^{\bullet-}$, H_2O_2, as well as other oxidants [93, 94]. Cancer cells are known to manifest enhanced intrinsic oxidative stress and metabolic activity that lead to mitochondrial failure [99, 100]. Indeed, it was previously reported that ovarian tumors are characterized by increased ROS levels as evident from increased $O_2^{\bullet-}$ generated from NAD(P)H oxidase as well as mitochondrial malfunction [101]. The NAD(P)H oxidase redox signaling is controlled by mitochondria, and thus loss of

this control is thought to contribute to tumorigenesis [101]. Others have also shown that inhibition of NAD(P)H oxidase induced apoptosis in cancer cells [102]. Continuous ROS production by the cell and the environment further induces the inhibition of phosphorylation of AKT and subsequent suppression of AKT-mediated phosphorylation of ASK1 on Ser-83, resulting in significant decrease in apoptosis [102–104]. Furthermore, paclitaxel, a chemotherapeutic agent used in the treatment of ovarian cancer and other cancers, induced apoptosis of ovarian cancer cells by negative regulation of AKT–ASK1 phosphorylation signaling [102–104]. On the other hand, activation of AKT by ROS provided protection against apoptosis [102–104].

Data from our laboratory clearly demonstrated that treatment of EOC cells with DPI, which inhibits ROS production mediated by NAD(P)H oxidase, significantly reduced SOD3 and HIF-1α mRNA and protein levels as early as 30 minutes after treatment with a concomitant increase in apoptosis [52]. The association between increased HIF-1α expression and decreased cellular apoptosis has also been demonstrated in lung and hepatoma cancer cells [94, 105]. Overexpression of HIF-1α is thought to decrease apoptosis by the upregulation of anti-apoptotic proteins, Bcl-2 and Bcl-xL and down regulation of pro-apoptotic proteins, BAX and BAK [106]. Inhibition of HIF-1α by rapamycin increased apoptosis by decreasing the expression of apoptosis inhibitor Bcl-2 in ovarian cancer xenografts [107]. Additionally, inhibition of HIF-1α by rapamycin enhanced apoptosis through the inhibition of cell survival signals in several other cell lines [107].

Most of the NAD(P)H oxidase-generated $O_2^{\bullet-}$ is utilized to produce H_2O_2 by nonenzymatic or SOD-catalyzed reactions [108–110]. Hydrogen peroxide serves as the precursor of more toxic hydroxyl radicals and thus is extremely destructive to cells and tissues [109–111]. The expression of SOD3 was reported to increase in response to intrinsic oxidative stress in ovarian cancer cells [112]. It has been demonstrated that overexpression of the SOD3 gene significantly suppressed lung cancer metastasis as well as inhibited the growth of B16-F1 melanoma tumors in mice [113, 114]. However, in a somewhat controversial study, it has been shown that inhibition of SOD selectively induced apoptosis of leukemia and ovarian cancer cells [10].

Under hypoxic conditions, SOD3 is overexpressed and has been reported to significantly induce the expression of HIF-1α in tumors through unknown mechanisms however, steady state levels of $O_2^{\bullet-}$ and the stabilization of HIF-1α have been proposed to play a role in this mechanism [107, 115]. Therefore, inhibition of NAD(P)H oxidase and the consequent reduction of $O_2^{\bullet-}$ levels may destabilize HIF-1α, and subsequently increase apoptosis by lowering SOD3 levels. Thus, we conclude that lowering oxidative stress, possibly through the inhibition of NAD(P)H oxidase-generated $O_2^{\bullet-}$, induces apoptosis in ovarian cancer cells and may serve as a potential target for cancer therapy. This effect was attributed to the modulation of key enzymes that are central to controlling the cellular redox balance.

8. Modulation of metabolism

Cancer cells are known to favor anaerobic metabolism, even when oxygen is present and is known as the "Warburg effect" [116, 117]. Aerobic glycolysis is known to decrease ATP yield as well as increase lactate production by cancer cells [116–118]. To compensate for this decrease in

ATP, cancer cells significantly increase glucose uptake through upregulation of glucose recep-tors [40, 41, 118]. Increased lactate in cancer cells enhances lactic acidosis, which is significantly toxic to the surrounding tissues and can facilitate tumor growth through the stimulation of ECM degradation, angiogenesis, and metastasis [118]. Additionally, aerobic glycolysis in can-cer cells activates HIF, an oxygen-sensitive transcription factor that plays an important role in initiation and maintenance of the oncogenic phenotype [118]. In this regard, HIF induces the expression of several glucose transporters and glycolysis enzymes as well as induces the expression of pyruvate dehydrogenate kinase (PDK), an enzyme that stimulates pyruvate entry into the mitochondria for oxidation [41, 118, 119]. Thus, shifting glucose metabolism in cancer cells from glycolysis to glucose oxidation may have therapeutic value [120]. Indeed, inhibiting PDK by dichloroacetate (DCA) has been reported to induce apoptosis in tumor cells and sig-nificantly decreased HIF-1α expression [40]. More importantly DCA is currently in the clinical use for the treatment of hereditary mitochondrial diseases as well as lactic acidosis [41, 121]. The use of DCA at a dose of 35 to 50 mg/kg decreased lactate levels by more than 60% [41, 122]. Dichloroacetate treatment has been shown to significantly induce apoptosis, through the stim-ulation of caspase-3 activity, in a dose-dependent manner in EOC cells as well as other cancers, such as glioblastoma, endometrial, prostate, and non-small cell lung cancers [40, 123]. Aerobic glycolysis is associated with resistance to apoptosis in cancer cells as many of the enzymes in the glycolysis process are known to modulate gene transcription of apoptotic proteins [40, 41, 69, 124]. Stimulation of pyruvate entry into the mitochondria by DCA, through activation of PDH and inhibition of PDK, is an ideal method to shift aerobic glycolysis to glucose oxidation as inhibiting aerobic glycolysis results in ATP depletion and necrosis, not apoptosis [41, 125].

An additional approach to induce apoptosis in cancer cells is through scavenging high levels of oxidants produced by cancer cells utilizing antioxidants [126]. Deficiency in SOD or inhibi-tion of SOD enzyme activity causes accumulation of $O_2^{\bullet-}$ which is the precursor for several toxic free radicals that are critical to the oncogenic process [127]. Elevated levels of oxidants and free radicals are also known to induce cellular senescence and necrosis, and thus can kill tumor cells [40, 128]. The precise effect of high levels of oxidants and free radicals in cancer cells will depend on the type of cells and tissues, the site of production, and the type and concentration of oxidants [13].

9. Chemotherapy and the acquisition of chemoresistance in EOC cells

Resistance to taxanes and platinums, chemotherapy drugs in current use for ovarian cancer treatment, remains a major obstacle to a successful treatment of ovarian cancer patients [6]. Resistance to chemotherapy not only limits the use of the initial drug but also limits the use of other agents, even those with different mechanisms of action [129]. Chemotherapy drugs exert their actions by the initiation of cell death either directly through the generation of oxidative stress or as an indirect effect of exposure, as observed with several chemotherapeutic agents [130]. The development of chemoresistance to drugs is dependent on several factors that include: influx/efflux of drugs that decrease platinum accumulation in tumor cells, enhanced GSH and GST levels, upregulation of anti-apoptotic proteins such as Bcl-2, loss of tumor necrosis factor receptor ligand which induces apoptosis, increased DNA repair through up-regulation of repair
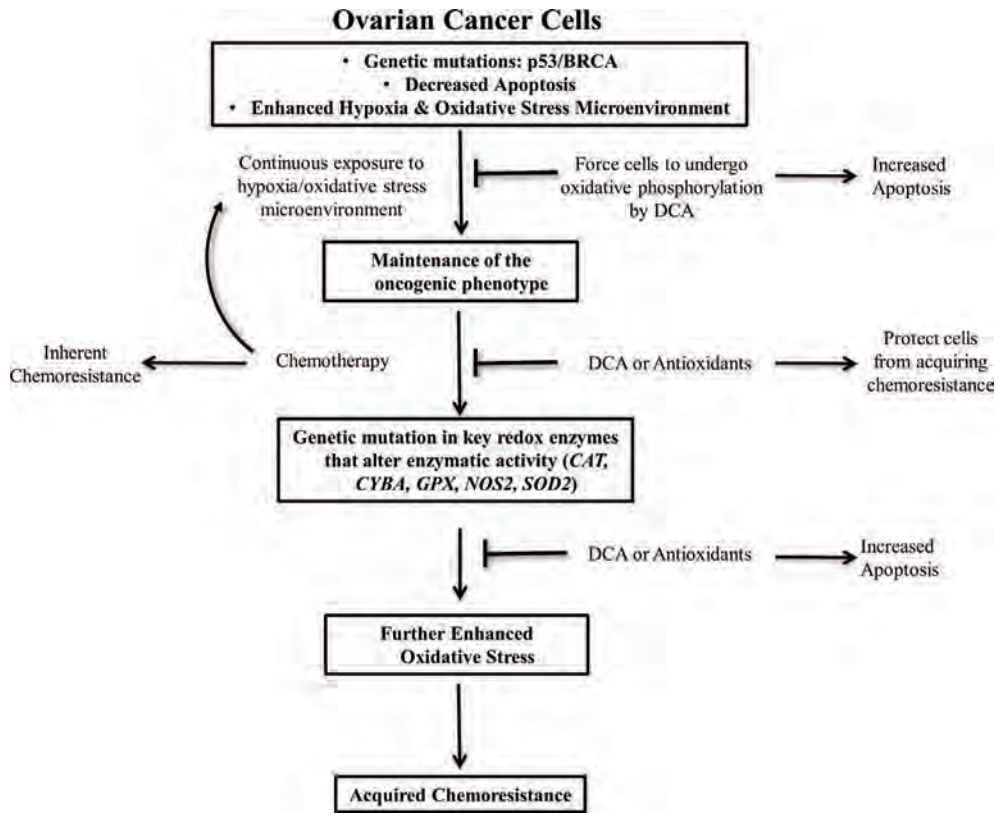
Figure 2. Summary of the role of oxidative stress in the development of sensitive and chemoresistant ovarian cancer [1].

genes, and loss of functional p53 that augments NF-κB activation [13, 131]. We have previously shown that chemoresistant EOC cells manifested increased iNOS and nitrate/nitrite levels as well as a decrease in GSR expression as compared to sensitive EOC cells, suggesting a further enhancement of the redox state in chemoresistant cells [1, 45]. Additionally, CAT, GPX, and iNOS were shown to be significantly increased while, GSR, SOD, and the NAD(P)H oxidase subunit (p22^{phox}) were decreased in chemoresistant EOC cells as compared to their sensitive counterparts [21]. These finding supports a key role for oxidative stress, not only in the development of the oncogenic phenotype, but also in the development of chemoresistance (**Figure 2**).

10. Common polymorphisms in redox enzymes are associated with ovarian cancer

A single nucleotide polymorphism (SNP) occurs as a result of gene point mutations with an estimated frequency of at least one in every 1000 base pairs that are selectively maintained and distributed in populations throughout the human genome [132]. An association

between common SNPs in oxidative DNA repair genes and redox genes with human cancer susceptibility has been established [28]. Common SNPs in the redox enzymes are known to be strongly associated with an altered enzymatic activity in these enzymes, and may explain the enhanced redox state that has been linked to several malignancies, including ovarian cancer [40, 52]. Additionally, it may further explain the observation of significantly decreased apoptosis and increased survival of EOC cells [53]. It is therefore critical to determine the exact effect of common SNPs in various redox enzymes on all process involved in the development of the oncogenic phenotype [21, 46, 133, 134]. Such studies can be linked to other studies focusing on determining the effects of genes involved in carcinogen metabolism (detoxification and/or activation), redox enzymes, and DNA repair pathways [133]. Numerous SNPs associated with change of function have been identified in antioxidant enzymes including *CAT*, *GPX1*, *GSR*, and *SOD2* [21, 134]. Additionally, the association between genetic polymorphisms in genes with anti-tumor activity and those involved in the cell cycle has been reported in ovarian cancer [135, 136]. Recently, several genetic variations have been identified in genome-wide association studies (GWAS), and were found to act as low to moderate penetrant alleles, which contribute to ovarian cancer risk, as well as other diseases [23, 137].

There is now an association of specific SNPs in key oxidant and anti-oxidant enzymes with increased risk and overall survival of ovarian cancer [21, 46]. A common SNP that reduced CAT activity (rs1001179) was utilized as a significant predictor of death when present in ovarian cancer patients and was also associated with increased risk for breast cancer [21, 46, 134, 138]. This SNP is also linked to increased risk, survival, and response to adjuvant treatment of cancer patients, including ovarian [46, 139]. Another common SNP that reduced CYBA activity (rs4673) was also reported to be associated with an increased risk for ovarian cancer [21, 46]. The mutant genotype of the *CYBA* gene has been shown to both decrease and increase activity of the protein, thereby altering the generation of $O_2^{\bullet-}$ [21, 46]. Moreover, functionally distinct *MPO* polymorphisms, such as (rs2333227) have been linked to relative increased risk for development of ovarian cancer as well as other cancers [21, 44, 46]. Additional SNPs that influenced the risk of EOC have been successfully identified from the GWAS studies including rs3814113 (located at 9p22, near BNC2), rs2072590 (located at 2q31, which contains a family of HOX genes), rs2665390 (located at 3q25, intronic to TIPARP), rs10088218 (located at 8q24, 700 kb downstream of MYC), rs8170 (located at 19p13, near MERIT40), and rs9303542 (located at 17q21, intronic to SKAP1) [21, 46]. Thus, the genetic component of increased ovarian cancer risk may be attributed to SNPs that result in point mutations in the redox genes and potentially other genes [140].

11. Chemoresistance is associated with point mutations in key redox enzymes in EOC cells

To date, the acquisition of chemoresistance in ovarian cancer is not fully understood. The enhanced oxidant state reported in chemoresistant EOC cells may be linked to point mutations in key redox enzymes [21]. Chemoresistant EOC cells manifested increased levels of CAT, GPX, and iNOS and decreased levels of GSR, SOD, and NAD(P)H oxidase as compared to their sensitive counterparts [21]. Interestingly, chemoresistant EOC cells, and not their sensitive counterparts,

manifested specific point mutations that corresponded to known functional SNPs, in key redox enzymes including *SOD2* (rs4880), *NOS2* (rs2297518), and *CYBA* (rs4673) [1]. However, altered enzymatic activity for CAT and GSR observed in chemoresistant EOC cells did not correspond to the specific SNP of interest in those enzymes, indicating involvement of other possible functional SNPs for those enzymes [21]. Coincidently, chemotherapy treatment induced point mutations that happen to correspond to known functional SNPs in key oxidant enzymes subsequently led to the acquisition of chemoresistance by EOC cells. Indeed, the induction of specific point mutations in *SOD2* or *GPX1* in sensitive EOC cells resulted in a decrease in the sensitivity to chemotherapy of these cells [21]. In fact, the addition of SOD to sensitive EOC cells during chemotherapy treatment synergistically increased the efficacy to chemotherapy [21].

Alternatively, the observed nucleotide switch in response to chemotherapy in EOC cells may be the result of nucleotide substitution, a process that includes transitions, replacement of one purine by the other or that of one pyrimidine by the other, or transversions, replacement of a purine by a pyrimidine or vice versa [21]. Indeed, hydroxyl radicals are known to react with DNA causing the formation of many pyrimidine and purine-derived lesions [21]. The oxidative damage to 8-Oxo-2′-deoxyguanosine, a major product of DNA oxidation, induces genetic alterations in oncogenes and tumor suppressor genes has been involved in tumor initiation and progression [21]. A GC to TA transversion has been reported in the *ras* oncogene and the *p53* tumor suppressor gene in several cancers. However, the GC to TA transversion is not unique to hydroxy-2′-deoxyguanosine, as CC to TT substitutions have been identified as signature mutations for oxidants and free radicals [21].

Moreover, the observed nucleotide switch in response to chemotherapy in EOC cells can be due to the fact that acquisition of chemoresistance generates an entirely different population of cells with a distinct genotype. Hence, chemotherapy kills the bulk of the tumor cells leaving a subtype of cancer cells with ability for repair and renewal, known as cancer stem cells (CSCs) [21]. Indeed, cancer stem cells have been isolated from various types of cancer including leukemia, breast, brain, pancreatic, prostate, ovarian and colon [21]. Interestingly, CSC populations were present in cultures of SKOV-3 EOC cells and have been shown to be chemoresistance in nature [21].

12. Further increasing pro-oxidant enzymes: potential survival mechanism

Apoptosis is a tightly regulated molecular process that removes excess or unwanted cells from organisms. Resistance to apoptosis is a key feature of cancer cells and is involved in the pathogenesis of cancer. We have previously reported that EOC cells have significantly increased levels of NO, which correlated with increased expression in iNOS [54]. We have also reported that EOC cells manifested lower apoptosis, which was markedly induced by inhibiting iNOS by L-NAME, indicating a strong link between apoptosis and NO/iNOS pathways in these cells [54]. Caspase-3 is known to play a critical role in controlling apoptosis, by participating in a cascade that is triggered in response to proapoptotic signals and culminates in cleavage of a set of

proteins, resulting in disassembly of the cell [141–144]. Caspase-3 was found to be S-nitrosylated on the catalytic-site cysteine in unstimulated human lymphocyte cell lines and denitrosylated upon activation of the Fas apoptotic pathway [145]. Decreased caspase-3 S-nitrosylation was associated with an increase in intracellular caspase activity. Caspase-3 S-nitrosylation/deni-trosylation is known to serve as an on/off switch regulating caspase activity during apopto-sis in endothelial cells, lymphocytes and trophoblasts [146–149]. The mechanisms underlying S-nitrosothiol (SNO) formation *in vivo* are not well understood.

Myeloperoxidase typically uses H_2O_2, in combination with chloride to generate hypochlorous acid [55, 150–153]. We, and others, have demonstrated that MPO utilizes NO, produced by iNOS, as a one-electron substrate generating NO^+, a labile nitrosating species that is rapidly hydrolyzed forming nitrite as end-product [55, 56, 154, 155]. The ability of MPO to generate NO^+, from NO, led us to believe that not only does MPO play a role in S-nitrosylation of cas-pase-3 in EOC cells, but also highlights a possible cross-talk between iNOS and MPO. Indeed, we observed that MPO is responsible for the S-nitrosylation of caspase-3, which led to the inhibition of caspase-3 in EOC cells. Silencing MPO gene expression induced apoptosis in EOC cells through a mechanism that involved S-nitrosylation of caspase-3 by MPO.

Molecular alterations that lead to apoptosis can be inhibited by S-nitrosylation of apoptotic proteins such as caspases. Thus, S-nitrosylation conveys a key influence of NO on apoptosis signaling and may act as a key regulator for apoptosis in cancer cells. It has been known that the effects of NO on apoptosis are not only stimulatory but may also be inhibitory. These para-doxical effects of NO on apoptosis seem to be influenced by several factors. It has been sug-gested that biological conditions, such as the redox state, concentration, exposure time and the combination with O_2, $O_2^{\bullet-}$ and other molecules, determines the net effect of NO on apoptosis [156]. Also, NO is implicated in both apoptotic and necrotic cell death depending on the NO chemistry and the cellular biological redox state [57, 156]. As described earlier, we have previ-ously demonstrated that the EOC cell lines, SKOV-3 and MDAH-2774, manifested lower apop-tosis and had significantly higher levels of NO due to the presence of elevated levels of iNOS [54, 157]. We have also reported significant levels of MPO expression, which was found to be co-localized with iNOS, in both EOC cell lines SKOV-3 and MDAH-2774 [53]. We have dem-onstrated that 65% of the invasive epithelial ovarian carcinoma specimens tested expressed MPO in the neoplastic cells. The co-localization of MPO and iNOS has been demonstrated by immunohistochemical studies in cytokine-treated human neutrophils and primary granules of activated leukocytes [158]. Both plasma levels and tissue expression of MPO in gynecologic malignancies were previously evaluated and it was found that gynecologic cancer patients had higher plasma MPO compared to control subjects [159]. Using immunostaining, it was also demonstrated that MPO expression was higher in cancer tissues compared to control [159].

We have now characterized chemoresistant EOC cells to manifest an even further increase in pro-oxidant enzymes including MPO, and NO, a surrogate for iNOS activity in conjunc-tion with a further increase in the S-nitrosylation of caspase-3 (*data not published*) and a con-current decrease in the level of apoptosis [21]. Thus, we hypothesized that the decrease in apoptosis observed in chemoresistant EOC cells is a consequence of a further increase in the degree of S-nitrosylation of caspase-3. Since resistance to apoptosis is a hallmark of tumor

growth, identifying mechanisms of this resistance such as S-nitrosylation may be a key in cancer progression and the development of chemoresistance. S-nitrosylation is reversible and seemingly a specific post-translational modification that regulates the activity of several signaling proteins. S-nitrosylation of the catalytic site cysteine in caspases serves as an on/off switch regulating caspase activity during apoptosis in endothelial cells, lymphocytes, and trophoblasts [147–149]. Targeting MPO may be a potential therapeutic intervention to reverse the resistance to apoptosis in sensitive and chemoresistant EOC cells.

13. Ovarian cancer immunotherapy and oxidative stress

It is well established that tumorigenic cells generate high levels of ROS to activate proximal signaling pathways that promote proliferation, survival and metabolic adaptation while also maintaining a high level of antioxidant activity to prevent buildup of ROS to levels that could induce cell death [160]. Moreover, there is evidence that ROS can act as secondary messengers in immune cells, which can lead to hyperactivation of inflammatory responses resulting in tissue damage and pathology [160]. Ovarian cancer is considered an ideal tumorigenic cancer because ovarian cancer cells have no negative impact on immune cells [161].

Effective immunotherapy for ovarian cancer is currently the focus of several investigations and clinical trials. Current immunotherapies for cancer treatment include therapeutic vaccines, cytokines, immune modulators, immune checkpoint inhibitors, and adoptive T cell transfer [162]. The discovery of a monoclonal antibodies (such as bevacizumab) directed against VEGF have been shown to improve progression free survival compared to cytotoxic chemotherapy alone was a major outcome of these clinical trials [163]. Other monoclonal antibodies currently approved for other cancers such as trastuzumab for breast cancer or cetuximab for colon cancer exhibited limited activity in ovarian cancer [163]. Several clinical trials are ongoing for the utilization of immune checkpoint blockade in ovarian cancer immune therapy [164]. Most recently tested were the programmed death (PD)-1 inhibitors, pembrolizumab and nivolumab, which showed a consistent response rate of 10–20% in phase 2 studies and then failed to improve outcomes in confirmatory trials [164]. Ultimately, larger phase 3 studies are needed to validate these findings for checkpoint inhibitors, particularly with regard to the duration of response seen with these agents. Additionally, the direct intraperitoneal delivery of interleukin (IL)-12, a potent immunostimulatory agent, exhibited some potential therapeutic efficacy in ovarian cancer [165]. Recently, targeting folate receptor alpha, which is found to be expressed in ovarian cancer, has shown promising therapeutic value. The targeting of the folate receptor was achieved by either a blocking monoclonal antibody (farletuzumab) or antibody conjugates of folate analogs, such as vintafolide [166].

14. Summary and conclusion

Oxidative stress has been implicated in the pathogenesis of several malignancies including ovarian cancer. Epithelial ovarian cancer is characterized to manifest a persistent pro-oxidant

state through alteration of the redox balance, which is further enhanced in their chemoresistant counterparts, as summarized in **Figure 2**. Forcing ovarian cancer cells to undergo oxidative phosphorylation rather than glycolysis has been shown to be beneficial for eliminating cells via apoptosis (**Figure 2**). Collectively, there is convincing evidence that indicated a causal relationship between the acquisition of chemoresistance and chemotherapy-induced genetic mutations in key redox enzymes, leading to a further enhanced oxidative stress in chemo-resistant EOC cells. This concept was further confirmed by the observation that induction of point mutations in sensitive EOC cells increased their resistance to chemotherapy. Also, a combination of antioxidants with chemotherapy significantly sensitized cells to chemotherapy. Identification of targets for chemoresistance with either biomarker and/or screening potential will have a significant impact for the treatment of this disease.

Acknowledgements

Author details

Ghassan M. Saed[1]*, Robert T. Morris[2] and Nicole M. Fletcher[1]

*Address all correspondence to: gsaed@med.wayne.edu

1 Wayne State University, Detroit, MI, USA

2 Karmanos Cancer Institute, Detroit, MI, USA

References

[1] Saed GM, Diamond MP, Fletcher NM. Updates of the role of oxidative stress in the pathogenesis of ovarian cancer. Gynecologic Oncology. 2017;**145**(3):595-602

[2] Rojas V et al. Molecular characterization of epithelial ovarian cancer: Implications for diagnosis and treatment. International Journal of Molecular Sciences. 2016;**17**(12)

[3] Blagden SP. Harnessing pandemonium: The clinical implications of tumor heterogeneity in ovarian cancer. Frontiers in Oncology. 2015;**5**:149

[4] Leahy Y. Are serum protein biomarkers effective in detecting ovarian cancer in its early stages? Clinical Journal of Oncology Nursing. 2009;**13**(4):443-445

[5] Zhen W et al. Increased gene-specific repair of cisplatin interstrand cross-links in cisplatin-resistant human ovarian cancer cell lines. Molecular and Cellular Biology. 1992;**12**(9):3689-3698

[6] Lee C, Macgregor P. Drug resistance and microarrays. Modern Drug Discovery. 2004:**7**(7)

[7] Lippert TH, Ruoff HJ, Volm M. Current status of methods to assess cancer drug resistance. International Journal of Medical Sciences. 2011;**8**(3):245-253

[8] Matsuo K et al. Clinical relevance of extent of extreme drug resistance in epithelial ovarian carcinoma. Gynecologic Oncology. 2010;**116**(1):61-65

[9] Taddei ML et al. Mitochondrial oxidative stress due to complex I dysfunction promotes fibroblast activation and melanoma cell invasiveness. Journal of Signal Transduction. 2012;**2012**:684592

[10] Hileman EO et al. Intrinsic oxidative stress in cancer cells: A biochemical basis for therapeutic selectivity. Cancer Chemotherapy and Pharmacology. 2004;**53**(3):209-219

[11] Toyokuni S. Oxidative stress and cancer: The role of redox regulation. Biotherapy. 1998;**11**(2-3):147-154

[12] Toyokuni S et al. Persistent oxidative stress in cancer. FEBS Letters. 1995;**358**(1):1-3

[13] Reuter S et al. Oxidative stress, inflammation, and cancer: How are they linked? Free Radical Biology & Medicine. 2010;**49**(11):1603-1616

[14] Beckman KB, Ames BN. The free radical theory of aging matures. Physiological Reviews. 1998;**78**(2):547-581

[15] Choi JY et al. Iron intake, oxidative stress-related genes (MnSOD and MPO) and prostate cancer risk in CARET cohort. Carcinogenesis. 2008;**29**(5):964-970

[16] Coussens LM, Werb Z. Inflammation and cancer. Nature. 2002;**420**(6917):860-867

[17] Finkel T, Holbrook NJ. Oxidants, oxidative stress and the biology of ageing. Nature. 2000;**408**(6809):239-247

[18] Erickson BK, Conner MG, Landen CN Jr. The role of the fallopian tube in the origin of ovarian cancer. American Journal of Obstetrics and Gynecology. 2013;**209**(5):409-414

[19] Kurman RJ, Shih Ie M. The dualistic model of ovarian carcinogenesis: Revisited, revised, and expanded. American Journal of Pathology. 2016;**186**(4):733-747

[20] Hibbs K et al. Differential gene expression in ovarian carcinoma: Identification of potential biomarkers. The American Journal of Pathology. 2004;**165**(2):397-414

[21] Fletcher NM et al. Specific point mutations in key redox enzymes are associated with chemoresistance in epithelial ovarian cancer. Free Radical Biology & Medicine. 2016;**102**:122-132

[22] Fletcher NM et al. Myeloperoxidase and free iron levels: Potential biomarkers for early detection and prognosis of ovarian cancer. Cancer Biomarkers. 2011;**10**(6):267-275

[23] Ramus SJ et al. Consortium analysis of 7 candidate SNPs for ovarian cancer. International Journal of Cancer. 2008;**123**(2):380-388

[24] Lengyel E. Ovarian cancer development and metastasis. The American Journal of Pathology. 2010;**177**(3):1053-1064

[25] Liao J et al. Ovarian cancer spheroid cells with stem cell-like properties contribute to tumor generation, metastasis and chemotherapy resistance through hypoxia-resistant metabolism. PLoS One. 2014;**9**(1) e84941

[26] Vermeersch KA et al. OVCAR-3 spheroid-derived cells display distinct metabolic profiles. PLoS One. 2015;**10**(2):e0118262

[27] Lei XG et al. Paradoxical roles of antioxidant enzymes: Basic mechanisms and health implications. Physiological Reviews. 2016;**96**(1):307-364

[28] Klaunig JE, Kamendulis LM, Hocevar BA. Oxidative stress and oxidative damage in carcinogenesis. Toxicologic Pathology. 2010;**38**(1):96-109

[29] Fruehauf JP, Meyskens FL Jr. Reactive oxygen species: A breath of life or death? Clinical Cancer Research. 2007;**13**(3):789-794

[30] Circu ML, Aw TY. Glutathione and modulation of cell apoptosis. Biochimica et Biophysica Acta. 2012;**1823**(10):1767-1777

[31] Ishikawa T, Ali-Osman F. Glutathione-associated cis-diamminedichloroplatinum(II) metabolism and ATP-dependent efflux from leukemia cells. Molecular characterization of glutathione-platinum complex and its biological significance. The Journal of Biological Chemistry. 1993;**268**(27):20116-20125

[32] Wang J, Yi J. Cancer cell killing via ROS: To increase or decrease, that is the question. Cancer Biology & Therapy. 2008;**7**(12):1875-1884

[33] Schmidt HH et al. Antioxidants in translational medicine. Antioxidants & Redox Signaling. 2015;**23**(14):1130-1143

[34] Waris G, Ahsan H. Reactive oxygen species: Role in the development of cancer and various chronic conditions. Journal of Carcinogenesis. 2006;**5**:14

[35] Roos WP, Thomas AD, Kaina B. DNA damage and the balance between survival and death in cancer biology. Nature Reviews. Cancer. 2016;**16**(1):20-33

[36] Retel J et al. Mutational specificity of oxidative DNA damage. Mutation Research. 1993;**299**(3-4):165-182

[37] Westermarck J, Kahari VM. Regulation of matrix metalloproteinase expression in tumor invasion. The FASEB Journal. 1999;**13**(8):781-792

[38] Mazure NM et al. Oncogenic transformation and hypoxia synergistically act to modulate vascular endothelial growth factor expression. Cancer Research. 1996;**56**(15):3436-3440

[39] Okada F et al. Impact of oncogenes in tumor angiogenesis: Mutant K-ras up-regulation of vascular endothelial growth factor/vascular permeability factor is necessary, but not sufficient for tumorigenicity of human colorectal carcinoma cells. Proceedings of the National Academy of Sciences of the United States of America. 1998;**95**(7):3609-3614

[40] Saed GM et al. Dichloroacetate induces apoptosis of epithelial ovarian cancer cells through a mechanism involving modulation of oxidative stress. Reproductive Sciences. 2011;**18**(12):1253-1261

[41] Michelakis ED, Webster L, Mackey JR. Dichloroacetate (DCA) as a potential metabolic-targeting therapy for cancer. British Journal of Cancer. 2008;**99**(7):989-994

[42] Bell EL, Emerling BM, Chandel NS. Mitochondrial regulation of oxygen sensing. Mitochondrion. 2005;**5**(5):322-332

[43] Mansfield KD et al. Mitochondrial dysfunction resulting from loss of cytochrome c impairs cellular oxygen sensing and hypoxic HIF-alpha activation. Cell Metabolism. 2005;**1**(6):393-399

[44] Castillo-Tong DC et al. Association of myeloperoxidase with ovarian cancer. Tumour Biology. 2014;**35**(1):141-148

[45] Belotte J et al. The role of oxidative stress in the development of cisplatin resistance in epithelial ovarian cancer. Reproductive Sciences. 2013

[46] Belotte J et al. A single nucleotide polymorphism in catalase is strongly associated with ovarian cancer survival. PLoS One. 2015;**10**(8):e0135739

[47] Watson J. Oxidants, antioxidants and the current incurability of metastatic cancers. Open Biology. 2013;**3**(1):120144

[48] Klein EA et al. Vitamin E and the risk of prostate cancer: The selenium and vitamin E cancer prevention trial (SELECT). JAMA. 2011;**306**(14):1549-1556

[49] Bjelakovic G et al. Mortality in randomized trials of antioxidant supplements for primary and secondary prevention: Systematic review and meta-analysis. JAMA. 2007;**297**(8):842-857

[50] Sayin VI et al. Antioxidants accelerate lung cancer progression in mice. Science Translational Medicine. 2014;**6**(221) 221ra15

[51] Senthil K, Aranganathan S, Nalini N. Evidence of oxidative stress in the circulation of ovarian cancer patients. Clinica Chimica Acta. 2004;**339**(1-2):27-32

[52] Jiang Z et al. Modulation of redox signaling promotes apoptosis in epithelial ovarian cancer cells. Gynecologic Oncology. 2011;**122**(2):418-423

[53] Saed GM et al. Myeloperoxidase serves as a redox switch that regulates apoptosis in epithelial ovarian cancer. Gynecologic Oncology. 2010;**116**(2):276-281

[54] Malone JM et al. The effects of the inhibition of inducible nitric oxide synthase on angiogenesis of epithelial ovarian cancer. American Journal of Obstetrics and Gynecology. 2006;**194**(4):1110-6; discussion 1116-8

[55] Abu-Soud HM, Hazen SL. Nitric oxide is a physiological substrate for mammalian peroxidases. The Journal of Biological Chemistry. 2000;**275**(48):37524-37532

[56] Abu-Soud HM, Hazen SL. Nitric oxide modulates the catalytic activity of myeloperoxidase. The Journal of Biological Chemistry. 2000;**275**(8):5425-5430

[57] Habib S, Ali A. Biochemistry of nitric oxide. Indian Journal of Clinical Biochemistry. 2011;**26**(1):3-17

[58] Muscat JE, Huncharek MS. Perineal talc use and ovarian cancer: A critical review. European Journal of Cancer Prevention. 2008;**17**(2):139-146

[59] Sasaroli D, Coukos G, Scholler N. Beyond CA125: The coming of age of ovarian cancer biomarkers. Are we there yet? Biomarkers in Medicine. 2009;**3**(3):275-288

[60] Schummer M et al. Comparative hybridization of an array of 21,500 ovarian cDNAs for the discovery of genes overexpressed in ovarian carcinomas. Gene. 1999;**238**(2):375-385

[61] Drapkin R et al. Human epididymis protein 4 (HE4) is a secreted glycoprotein that is overexpressed by serous and endometrioid ovarian carcinomas. Cancer Research. 2005;**65**(6):2162-2169

[62] Galgano MT, Hampton GM, Frierson HF Jr. Comprehensive analysis of HE4 expression in normal and malignant human tissues. Modern Pathology. 2006;**19**(6):847-853

[63] Gilks CB et al. Distinction between serous tumors of low malignant potential and serous carcinomas based on global mRNA expression profiling. Gynecologic Oncology. 2005;**96**(3):684-694

[64] Hough CD et al. Large-scale serial analysis of gene expression reveals genes differentially expressed in ovarian cancer. Cancer Research. 2000;**60**(22):6281-6287

[65] Bouchard D et al. Proteins with whey-acidic-protein motifs and cancer. The Lancet Oncology. 2006;**7**(2):167-174

[66] Rosen DG et al. Potential markers that complement expression of CA125 in epithelial ovarian cancer. Gynecologic Oncology. 2005;**99**(2):267-277

[67] Scholler N et al. Bead-based ELISA for validation of ovarian cancer early detection markers. Clinical Cancer Research. 2006;**12**(7 Pt 1):2117-2124

[68] Moore RG et al. The use of multiple novel tumor biomarkers for the detection of ovarian carcinoma in patients with a pelvic mass. Gynecologic Oncology. 2008;**108**(2):402-408

[69] Kim JW, Dang CV. Multifaceted roles of glycolytic enzymes. Trends in Biochemical Sciences. 2005;**30**(3):142-150

[70] Menon U et al. Prospective study using the risk of ovarian cancer algorithm to screen for ovarian cancer. Journal of Clinical Oncology. 2005;**23**(31):7919-7926

[71] Xu FJ et al. OVX1 as a marker for early stage endometrial carcinoma. Cancer. 1994;**73**(7):1855-1858

[72] Havrilesky LJ et al. Evaluation of biomarker panels for early stage ovarian cancer detection and monitoring for disease recurrence. Gynecologic Oncology. 2008;**110**(3):374-382

[73] Loria V et al. Myeloperoxidase: A new biomarker of inflammation in ischemic heart disease and acute coronary syndromes. Mediators of Inflammation. 2008;**2008**:135625

[74] Dally H et al. Myeloperoxidase (MPO) genotype and lung cancer histologic types: The MPO -463 a allele is associated with reduced risk for small cell lung cancer in smokers. International Journal of Cancer. 2002;**102**(5):530-535

[75] Ekmekci OB et al. Iron, nitric oxide, and myeloperoxidase in asthmatic patients. Biochemistry (Mosc). 2004;**69**(4):462-467

[76] Baldus S et al. Myeloperoxidase serum levels predict risk in patients with acute coronary syndromes. Circulation. 2003;**108**(12):1440-1445

[77] Brennan ML et al. Prognostic value of myeloperoxidase in patients with chest pain. The New England Journal of Medicine. 2003;**349**(17):1595-1604

[78] Muscara MN et al. Wound collagen deposition in rats: Effects of an NO-NSAID and a selective COX-2 inhibitor. British Journal of Pharmacology. 2000;**129**(4):681-686

[79] Shi HP et al. The role of iNOS in wound healing. Surgery. 2001;**130**(2):225-229

[80] Witte MB, Barbul A. Role of nitric oxide in wound repair. American Journal of Surgery. 2002;**183**(4):406-412

[81] Sadrzadeh SM et al. Hemoglobin. A biologic fenton reagent. The Journal of Biological Chemistry. 1984;**259**(23):14354-14356

[82] Galijasevic S et al. Myeloperoxidase interaction with peroxynitrite: Chloride deficiency and heme depletion. Free Radical Biology & Medicine. 2009;**47**(4):431-439

[83] Maitra D et al. Melatonin can mediate its vascular protective effect by modulating free iron level by inhibiting hypochlorous acid-mediated hemoprotein heme destruction. Hypertension. 2011;**57**(5):e22 author reply e23

[84]   Maitra D et al. Reaction of hemoglobin with HOCl: Mechanism of heme destruction and free iron release. Free Radical Biology & Medicine. 2011;**51**(2):374-386

[85]   Maitra D et al. Mechanism of hypochlorous acid-mediated heme destruction and free iron release. Free Radical Biology & Medicine. 2011;**51**(2):364-373

[86]   Bozzini C et al. Biochemical and genetic markers of iron status and the risk of coronary artery disease: An angiography-based study. Clinical Chemistry. 2002;**48**(4):622-628

[87]   de Valk B, Marx JJ. Iron, atherosclerosis, and ischemic heart disease. Archives of Internal Medicine. 1999;**159**(14):1542-1548

[88]   Sullivan JL. Iron and the genetics of cardiovascular disease. Circulation. 1999;**100**(12): 1260-1263

[89]   Niederau C. Iron overload and atherosclerosis. Hepatology. 2000;**32**(3):672-674

[90]   Brault S et al. Lysophosphatidic acid induces endothelial cell death by modulating the redox environment. American Journal of Physiology. Regulatory, Integrative and Comparative Physiology. 2007;**292**(3):R1174-R1183

[91]   O'Donnell BV et al. Studies on the inhibitory mechanism of iodonium compounds with special reference to neutrophil NADPH oxidase. The Biochemical Journal. 1993;**290** (Pt 1):41-49

[92]   Park SE et al. Diphenyleneiodonium induces ROS-independent p53 expression and apoptosis in human RPE cells. FEBS Letters. 2007;**581**(2):180-186

[93]   Boveris A, Chance B. The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen. The Biochemical Journal. 1973;**134**(3):707-716

[94]   Volm M, Koomagi R. Hypoxia-inducible factor (HIF-1) and its relationship to apoptosis and proliferation in lung cancer. Anticancer Research. 2000;**20**(3A):1527-1533

[95]   Tanaka M et al. Anti-metastatic gene therapy utilizing subcutaneous inoculation of EC-SOD gene transduced autologous fibroblast suppressed lung metastasis of meth-a cells and 3LL cells in mice. Gene Therapy. 2001;**8**(2):149-156

[96]   Wheeler MD, Smutney OM, Samulski RJ. Secretion of extracellular superoxide dis-mutase from muscle transduced with recombinant adenovirus inhibits the growth of B16 melanomas in mice. Molecular Cancer Research. 2003;**1**(12):871-881

[97]   Suliman HB, Ali M, Piantadosi CA. Superoxide dismutase-3 promotes full expression of the EPO response to hypoxia. Blood. 2004;**104**(1):43-50

[98]   Arbiser JL et al. Reactive oxygen generated by Nox1 triggers the angiogenic switch. Proceedings of the National Academy of Sciences of the United States of America. 2002; **99**(2):715-720

[99]   Pelicano H, Carney D, Huang P. ROS stress in cancer cells and therapeutic implications. Drug Resistance Updates. 2004;**7**(2):97-110

[100] Trachootham D, Alexandre J, Huang P. Targeting cancer cells by ROS-mediated mechanisms: A radical therapeutic approach? Nature Reviews. Drug Discovery. 2009;**8**(7):579-591

[101] Desouki MM et al. Cross talk between mitochondria and superoxide generating NADPH oxidase in breast and ovarian tumors. Cancer Biology & Therapy. 2005;**4**(12):1367-1373

[102] Mochizuki T et al. Inhibition of NADPH oxidase 4 activates apoptosis via the AKT/apoptosis signal-regulating kinase 1 pathway in pancreatic cancer PANC-1 cells. Oncogene. 2006;**25**(26):3699-3707

[103] Mabuchi S et al. Estrogen inhibits paclitaxel-induced apoptosis via the phosphorylation of apoptosis signal-regulating kinase 1 in human ovarian cancer cell lines. Endocrinology. 2004;**145**(1):49-58

[104] Wang X et al. Epidermal growth factor receptor-dependent Akt activation by oxidative stress enhances cell survival. The Journal of Biological Chemistry. 2000;**275**(19):14624-14631

[105] Piret JP et al. CoCl2, a chemical inducer of hypoxia-inducible factor-1, and hypoxia reduce apoptotic cell death in hepatoma cell line HepG2. Annals of the New York Academy of Sciences. 2002;**973**:443-447

[106] Li Y, Trush MA. Diphenyleneiodonium, an NAD(P)H oxidase inhibitor, also potently inhibits mitochondrial reactive oxygen species production. Biochemical and Biophysical Research Communications. 1998;**253**(2):295-299

[107] Shi Y et al. Rapamycin enhances apoptosis and increases sensitivity to cisplatin in vitro. Cancer Research. 1995;**55**(9):1982-1988

[108] Scaife RM. Selective and irreversible cell cycle inhibition by diphenyleneiodonium. Molecular Cancer Therapeutics. 2005;**4**(6):876-884

[109] Goud AP et al. Reactive oxygen species and oocyte aging: Role of superoxide, hydrogen peroxide, and hypochlorous acid. Free Radical Biology & Medicine. 2008;**44**(7):1295-1304

[110] McCord JM, Fridovich I. Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). The Journal of Biological Chemistry. 1969;**244**(22):6049-6055

[111] Schallreuter KU et al. In vivo and in vitro evidence for hydrogen peroxide (H2O2) accumulation in the epidermis of patients with vitiligo and its successful removal by a UVB-activated pseudocatalase. The Journal of Investigative Dermatology. Symposium Proceedings. 1999;**4**(1):91-96

[112] Hu Y et al. Mitochondrial manganese-superoxide dismutase expression in ovarian cancer: Role in cell proliferation and response to oxidative stress. The Journal of Biological Chemistry. 2005;**280**(47):39485-39492

[113] Calastretti A et al. Damaged microtubules can inactivate BCL-2 by means of the mTOR kinase. Oncogene. 2001;**20**(43):6172-6180

[114] Jiang H, Feng Y. Hypoxia-inducible factor 1alpha (HIF-1alpha) correlated with tumor growth and apoptosis in ovarian cancer. International Journal of Gynecological Cancer. 2006;**16**(Suppl 1):405-412

[115] Kaewpila S et al. Manganese superoxide dismutase modulates hypoxia-inducible factor-1 alpha induction via superoxide. Cancer Research. 2008;**68**(8):2781-2788

[116] Chen B et al. Roles of microRNA on cancer cell metabolism. Journal of Translational Medicine. 2012;**10**:228

[117] Vander Heiden MG et al. Evidence for an alternative glycolytic pathway in rapidly proliferating cells. Science. 2010;**329**(5998):1492-1499

[118] Kroemer G, Pouyssegur J. Tumor cell metabolism: cancer's Achilles' heel. Cancer Cell. 2008;**13**(6):472-482

[119] Kim JW et al. HIF-1-mediated expression of pyruvate dehydrogenase kinase: A metabolic switch required for cellular adaptation to hypoxia. Cell Metabolism. 2006;**3**(3):177-185

[120] Xie J et al. Dichloroacetate shifts the metabolism from glycolysis to glucose oxidation and exhibits synergistic growth inhibition with cisplatin in HeLa cells. International Journal of Oncology. 2011;**38**(2):409-417

[121] Stacpoole PW et al. Evaluation of long-term treatment of children with congenital lactic acidosis with dichloroacetate. Pediatrics. 2008;**121**(5):e1223-e1228

[122] Stacpoole PW. The pharmacology of dichloroacetate. Metabolism. 1989;**38**(11):1124-1144

[123] Stockwin LH et al. Sodium dichloroacetate selectively targets cells with defects in the mitochondrial ETC. International Journal of Cancer. 2010;**127**(11):2510-2519

[124] Kim JW, Dang CV. Cancer's molecular sweet tooth and the Warburg effect. Cancer Research. 2006;**66**(18):8927-8930

[125] Xu RH et al. Inhibition of glycolysis in cancer cells: A novel strategy to overcome drug resistance associated with mitochondrial respiratory defect and hypoxia. Cancer Research. 2005;**65**(2):613-621

[126] Kinnula VL, Crapo JD. Superoxide dismutases in malignant cells and human tumors. Free Radical Biology & Medicine. 2004;**36**(6):718-744

[127] Tandon R et al. Oxidative stress in patients with essential hypertension. National Medical Journal of India. 2005;**18**(6):297-299

[128] Storz P. Reactive oxygen species in tumor progression. Frontiers in Bioscience. 2005;**10**:1881-1896

[129] Kajiyama H et al. Survival benefit of taxane plus platinum in recurrent ovarian cancer with non-clear cell, non-mucinous histology. Journal of Gynecologic Oncology. 2014;**25**(1):43-50

[130] Landriscina M et al. Adaptation to oxidative stress, chemoresistance, and cell survival. Antioxidants & Redox Signaling. 2009;**11**(11):2701-2716

[131] Traverso N et al. Role of glutathione in cancer progression and chemoresistance. Oxidative Medicine and Cellular Longevity. 2013;**2013**:972913

[132] Erichsen HC, Chanock SJ. SNPs in cancer research and treatment. British Journal of Cancer. 2004;**90**(4):747-751

[133] Klaunig JE et al. Oxidative stress and oxidative damage in chemical carcinogenesis. Toxicology and Applied Pharmacology. 2011;**254**(2):86-99

[134] Forsberg L et al. A common functional C-T substitution polymorphism in the promoter region of the human catalase gene influences transcription factor binding, reporter gene transcription and is correlated to blood catalase levels. Free Radical Biology & Medicine. 2001;**30**(5):500-505

[135] Goode EL et al. Candidate gene analysis using imputed genotypes: Cell cycle single-nucleotide polymorphisms and ovarian cancer risk. Cancer Epidemiology, Biomarkers & Prevention. 2009;**18**(3):935-944

[136] Notaridou M et al. Common alleles in candidate susceptibility genes associated with risk and development of epithelial ovarian cancer. International Journal of Cancer. 2011; **128**(9):2063-2074

[137] Savas S et al. Functional nsSNPs from carcinogenesis-related genes expressed in breast tissue: Potential breast cancer risk alleles and their distribution across human populations. Human Genomics. 2006;**2**(5):287-296

[138] Quick SK et al. Effect modification by catalase genotype suggests a role for oxidative stress in the association of hormone replacement therapy with postmenopausal breast cancer risk. Cancer Epidemiology, Biomarkers & Prevention. 2008;**17**(5):1082-1087

[139] Didziapetriene J et al. Significance of blood serum catalase activity and malondialdehyde level for survival prognosis of ovarian cancer patients. Medicina (Kaunas, Lithuania). 2014;**50**(4):204-208

[140] Sellers TA et al. Association of single nucleotide polymorphisms in glycosylation genes with risk of epithelial ovarian cancer. Cancer Epidemiology, Biomarkers & Prevention. 2008;**17**(2):397-404

[141] Porter AG, Janicke RU. Emerging roles of caspase-3 in apoptosis. Cell Death and Differentiation. 1999;**6**(2):99-104

[142] Liu L, Stamler JS. NO: An inhibitor of cell death. Cell Death and Differentiation. 1999;**6**(10):937-942

[143] Dimmeler S et al. Suppression of apoptosis by nitric oxide via inhibition of interleukin-1beta-converting enzyme (ICE)-like and cysteine protease protein (CPP)-32-like proteases. The Journal of Experimental Medicine. 1997;**185**(4):601-607

[144] Thornberry NA, Lazebnik Y. Caspases: Enemies within. Science. 1998;**281**(5381):1312-1316

[145] Mannick JB et al. Fas-induced caspase denitrosylation. Science. 1999;**284**(5414):651-654

[146] Maejima Y et al. Nitric oxide inhibits myocardial apoptosis by preventing caspase-3 activity via S-nitrosylation. Journal of Molecular and Cellular Cardiology. 2005; **38**(1):163-174

[147] Rossig L et al. Nitric oxide inhibits caspase-3 by S-nitrosation in vivo. The Journal of Biological Chemistry. 1999;**274**(11):6823-6826

[148] Dash PR et al. Nitric oxide protects human extravillous trophoblast cells from apoptosis by a cyclic GMP-dependent mechanism and independently of caspase 3 nitrosylation. Experimental Cell Research. 2003;**287**(2):314-324

[149] Mannick JB et al. S-Nitrosylation of mitochondrial caspases. The Journal of Cell Biology. 2001;**154**(6):1111-1116

[150] Harrison JE, Schultz J. Studies on the chlorinating activity of myeloperoxidase. The Journal of Biological Chemistry. 1976;**251**(5):1371-1374

[151] Kettle AJ, van Dalen CJ, Winterbourn CC. Peroxynitrite and myeloperoxidase leave the same footprint in protein nitration. Redox Report. 1997;**3**(5-6):257-258

[152] Weiss SJ et al. Chlorination of taurine by human neutrophils. Evidence for hypochlorous acid generation. Journal of Clinical Investigation. 1982;**70**(3):598-607

[153] Ortiz de Montellano PR. Catalytic sites of hemoprotein peroxidases. Annual Review of Pharmacology and Toxicology. 1992;**32**:89-107

[154] Stamler JS, Singel DJ, Loscalzo J. Biochemistry of nitric oxide and its redox-activated forms. Science. 1992;**258**(5090):1898-1902

[155] Abu-Soud HM et al. Peroxidases inhibit nitric oxide (NO) dependent bronchodilation: Development of a model describing NO-peroxidase interactions. Biochemistry. 2001;**40**(39):11866-11875

[156] Nicotera P, Melino G. Regulation of the apoptosis-necrosis switch. Oncogene. 2004;**23**(16): 2757-2765

[157] Munkarah AR et al. Effects of prostaglandin E(2) on proliferation and apoptosis of epithelial ovarian cancer cells. Journal of the Society for Gynecologic Investigation. 2002; **9**(3):168-173

[158] Evans TJ et al. Cytokine-treated human neutrophils contain inducible nitric oxide synthase that produces nitration of ingested bacteria. Proceedings of the National Academy of Sciences of the United States of America. 1996;**93**(18):9553-9558

[159] Song M, Santanam N. Increased myeloperoxidase and lipid peroxide-modified protein in gynecological malignancies. Antioxidants & Redox Signaling. 2001;**3**(6):1139-1146

[160] Schieber M, Chandel NS. ROS function in redox signaling and oxidative stress. Current Biology. 2014;**24**(10):R453-R462

[161] Kandalaft LE et al. Immunotherapy for ovarian cancer: what's next? Journal of Clinical Oncology. 2011;**29**(7):925-933

[162] De Felice F et al. Immunotherapy of ovarian cancer: The role of checkpoint inhibitors. Journal of Immunology Research. 2015;**2015**:191832

[163] Zand B, Coleman RL, Sood AK. Targeting angiogenesis in gynecologic cancers. Hematology/Oncology Clinics of North America. 2012;**26**(3):543-563 viii

[164] Chester C et al. Immunotherapeutic approaches to ovarian cancer treatment. Journal of Immunotherapy Cancer. 2015;**3**:7

[165] Alvarez RD et al. A phase II trial of intraperitoneal EGEN-001, an IL-12 plasmid formulated with PEG-PEI-cholesterol lipopolymer in the treatment of persistent or recurrent epithelial ovarian, fallopian tube or primary peritoneal cancer: A gynecologic oncology group study. Gynecologic Oncology. 2014;**133**(3):433-438

[166] Marchetti C et al. Targeted drug delivery via folate receptors in recurrent ovarian cancer: A review. Oncology Targets Therapy. 2014;**7**:1223-1236

Exhibit 82

Journal of Translational Medicine

BioMed Central

Review

Peritoneal inflammation – A microenvironment for Epithelial Ovarian Cancer (EOC)

Ralph S Freedman*[1], Michael Deavers[2], Jinsong Liu[2] and Ena Wang[3]

Address: [1]Department of Gynecologic Oncology, The University of Texas M.D. Anderson Cancer Center, Houston, TX, USA, [2]Department of Pathology, The University of Texas M.D. Anderson Cancer Center, Houston, TX, USA and [3]Department of Transfusion Medicine, NIH, Bethesda, MD, USA

Email: Ralph S Freedman* - rfreedma@mdanderson.org; Michael Deavers - mdeavers@mdanderson.org; Jinsong Liu - jliu@mdanderson.org; Ena Wang - EWang@cc.nih.gov

* Corresponding author

Abstract

Epithelial ovarian cancer (EOC) is a significant cause of cancer related morbidity and mortality in women. Preferential involvement of peritoneal structures contributes to the overall poor outcome in EOC patients. Advances in biotechnology, such as cDNA microarray, are a product of the Human Genome Project and are beginning to provide fresh opportunities to understand the biology of EOC. In particular, it is now possible to examine in depth, at the molecular level, the complex relationship between the tumor itself and its surrounding microenvironment.

This review focuses on the anatomy, physiology, and current immunobiologic research of peritoneal structures, and addresses certain potentially useful animal models. Changes in both the inflammatory and non-inflammatory cell compartments, as well as alterations to the extracellular matrix, appear to be signal events that contribute to the remodeling effects of the peritoneal stroma and surface epithelial cells on tumor growth and spread. These alterations may involve a number of proteins, including cytokines, chemokines, growth factors, either membrane or non-membrane bound, and integrins. Interactions between these molecules and molecular structures within the extracellular matrix, such as collagens and the proteoglycans, may contribute to a peritoneal mesothelial surface and stromal environment that is conducive to tumor cell proliferation and invasion. These alterations need to be examined and defined as possible prosnosticators and as therapeutic or diagnostic targets.

The peritoneum and its structures are integral to the microenvironment of epithelial ovarian cancer (EOC). The peritoneum comprises a single layer of mesothelial cells at the surface, covering abdominal organs (visceral or serosal layer) and the abdominal and pelvic wall (parietal layer or peritoneum).

About 80% of the more common epithelial ovarian cancers (EOC) involve the peritoneum or serosal surfaces as microscopic foci and visible lesions. The metastases may be exophytic with direct exposure to the peritoneal cavity and its contents or subperitoneal foci coalescing over time to form variably sized plaque-like deposits (Figure 1). Involvement of the peritoneum predicates an adverse situation for the patient that impacts significantly on prognosis as evidenced by the fact that Stage I patients have a 5 and 10 year survival of 90% [1], whereas patients with Stages III and IV disease have a 5 year survival of about
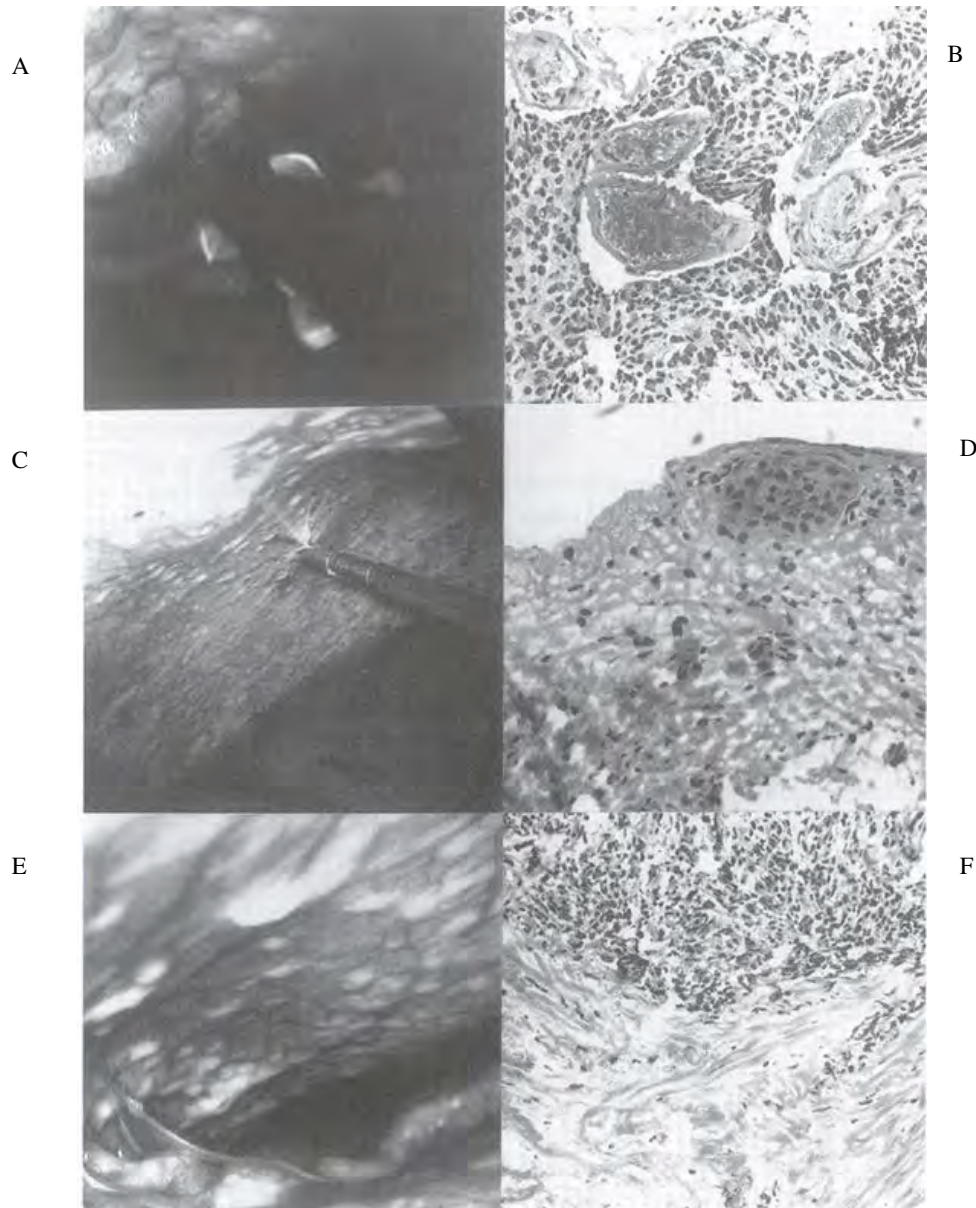
Figure 1
Surgical restaging by laparoscopy (peritonoscopy) and histopathologic findings showing different patterns of peritoneal involvement following prior systemic chemotherapy. (A,B) Exophytic peritoneal metastases approximately 1 cm in diameter showing multiple capillary loops. Histologic evaluation demonstrates numerous blood vessels surrounded by tumor cells. (C,D) Small 1 mm sized peritoneal metastases that are subperitoneal on histologic evaluation. (E,F) Multiple metastases about 1 cm in diameter that are growing deep to the peritoneal surface and coalescing to form plaques. Histologic evaluation demonstrates that these lesions are relatively avascular and contain significant amounts of peritumoral fibrosis. (Re-printed with permission from *Cytokines, Cellular & Molecular Therapy*).

http://www.translational-medicine.com/content/2/1/23

20%. Though most patients presenting with advanced disease show an initial response to chemotherapy, their fates are ultimately dependent upon sensitivity or resistance to chemotherapy agents or other factors. The important contributions of the tumor microenvironment to the malignant phenotype has been demonstrated in recent preclinical tumor models [2-4]. Findings from a recent study of human EOC tumors also suggest the possibility of genomic instability in nontumor tissues adjacent to growing tumor foci in EOC patients [5]. The current review summarizes the structural and functional components of the peritoneum, which could facilitate tumor progression and metastasis.

Anatomy and physiology of the peritoneum
The peritoneum has the structure and functions of an organ that is organized for the protection of the integrity of other abdominal organs and viscera. The surface epithelium of the serous membrane of the peritoneum and serosa, is attached to a basement membrane lying on a stroma of variable thickness, and is comprised of collagen-based matrix, blood vessels, lymphatics, nerve fibers, and, in the normal state, rare hematogenous cells. A detailed description of the micro and ultra structural anatomy is described elsewhere [6,7]. The structural and functional configuration of the peritoneum allows for an important homeostatic role through rapid mobilization of inflammatory mechanisms that can efficiently localize an injury or infection. The peritoneal surface layer has spaces or stomata between the surface mesothelial cells that could readily allow transfer of molecules or possibly cells between the stroma and the peritoneal cavity, or vice versa.

Immunohistochemically documented structures in the submesothelial layer include Type I and III collagen, fibronectin, elastin, and laminin at the basement membrane stromal interface and glycosaminoglycans [6]. Epithelial inclusions, referred to as endosalpingosis, can occur, though its cause is unknown. Ultrastructurally, both tight junctions and intercellular spaces are present. The presence of these junctions can affect the transfer of particles or cells. Molecules may transit either across or between the cells to the stromal compartment and vice versa.

Pathology and altered function of peritoneum in EOC
Peritoneal and serosal seeding is a frequent occurrence in EOC, but there is little known about the role of the multistructured peritoneum in contributing to invasion, metastasis, and tumor proliferation. It is possible, if not probable, that critical alterations in the peritoneum surface and stroma precede either lymphatic or hematogenous spread to distant sites. In EOC patients, there may be substantial alterations to the peritoneum both at the macroscopic and submacroscopic levels. Such alterations may include thickening of the surface membrane with or without malignant ascites formation and overtly enhanced vascularity. In certain patients, the peritoneum may have a florid appearance of peritonitis with edema, enhanced vascularity, and soft adhesions. At the microscopic level, there may be multilayering of the surface epithelium (hyperplasia) and an inflammatory infiltrate comprised of different leukocyte populations. Retroperitoneal fibrosis can be extensive and can interfere mechanically with anatomic structures in extraperitoneal locations, including ureters, lymphatics, and the bowel in different locations.

It would appear that a reorganization of the collagen-based matrix associated with the malignant process in EOC patients might accompany an inflammatory cell reaction. This could be similar to the situation in the peritoneum of renal fibrosis, which results in hyperplasia of the surface layer and extensive macrophage infiltrates into the stroma [8,9]. Since the peritoneal and serosal membranes lie in proximity to the primary tumor or its metastases, the question could be asked whether soluble products of tumor masses and nodules might transfer to the normal surface mesothelial cells and penetrate the subjacent stromal tissues. Molecules such as cytokines or chemokines released from the tumor into the peritoneal cavity could possibly prime these tissues for tumor spread, proliferation, and metastasis. The peritoneum can easily permit transperitoneal passage of molecules, even up to the size of albumin, and, depending on their adherence and reactivity with stromal compartment structures, might either transfer to the capillary bed or accumulate in the subperitoneal compartment, with later entry to the lymphatics. The dynamics of molecule transfer across the peritoneum as it applies to intraperitoneal therapy pharmacology and pharmacodynamics are reviewed elsewhere [10]. Peritoneal membrane and stromal structures exhibiting adherence properties for such molecules or cells, however, might retard their removal from this site, contributing to a sensitizing effect on the peritoneum.

Dual role of the inflammatory reactions in EOC
There is substantial data to support the presence of immune cell infiltration in EOC and its microenvironment. In earlier studies, we had shown that T cells comprised about 70% of mononuclear leukocytes in solid EOC tumors [11], and results from a number of experiments by us and by others suggested that the presence of these T cells could be associated with an antigen-driven immune response [12-17]. This effect is supported by the presence of clonally expanded T cell transcripts in ovarian TIL [18]. Notwithstanding these findings, we [19] and others [20] have found little evidence for the presence of an active ongoing adaptive immunity *in vivo*. This is

http://www.translational-medicine.com/content/2/1/23

supported by the absence of IFNγ transcripts in solid tumors and their infrequent detection in ascitic T cells [19]. Others have also reported absent CD3 TCRζ on TIL [20], and absent or low levels of IFNγ protein detected in ascites of EOC [21]. It is possible that cloned T cells in the tumor environment could represent tolerized cells, though antitumor activity can be generated ex vivo when these T cells are exposed to appropriate activation stimuli [13,14]. The presence of regulatory T cells [22], and certain macrophages [23], which are producers of IL6, IL10, and TGFβ could favor an immunosuppressive environment and may contribute to tumor progression and metastases [24]. The role of IL10 remains to be elucidated as, depending on the status of the tumor, this cytokine can either enhance or suppress immune responses [25].

Large numbers of monocyte/macrophages (MOMA) are also present in ascitic fluid where they may comprise 50% or more of the mononuclear leukocyte population, whereas the proportion of T-lymphocytes is usually below 40% [11]. In recent preliminary studies, we have found that pelvic peritoneal biopsies from advanced stage EOC patients, even in the absence of tumor involvement of the specimens, also has a high proportion of MO/MA. The MO/MA in EOC comprise several subsets with the notable presence of CD14+DR- and CD14+DR+ CD16+ cells [26]. The differentiation potential and functional capacity of these MO/MA in cancer patients is largely undetermined but clearly there are differences in the phenotypic characteristics between normal and EOC patients [26]. Inflammatory infiltrates have long been observed in human cancer tissues, but their significance in the non-lymphomatous solid tumors has largely been ignored by pathologists and clinicians. There is increasing recognition that infiltrating immune cells may contribute to either enhancement of immunity or tumor growth and progression [24]. Both MO/MA and T cells may have this dual role, and it remains a challenge to steer the activity of these populations toward an effective antitumor response *in vivo*.

We have recently employed a custom-made standardized cDNA microarray that utilizes probes for 16,500 genes to conduct a pilot study on the peritoneum of patients with EOC [27]. Biopsies were obtained at the time of peritoneal entry on patients undergoing exploratory surgery for suspected EOC. For controls, we utilized specimens of parietal peritoneum obtained at the same sites from patients undergoing surgery for suspected benign disease. Results from this study showed that the superficial layer, including the surface peritoneum and subjacent stroma specimens from the malignant group, revealed unique features at the transcript level compared to the benign group. These features are characterized by a dynamic process including cell attachments, signaling, growth stimula-

tion, and, most importantly, a proinflammatory, pro-angiogenic, and extracellular matrix (ECM) remodeling effects. The peritoneum and subperitoneal stroma from the benign cases showed homogeneity in their transcript expression without the proinflammatory signature contrasting with some heterogeneity from patients with EOC, but an emphasis on inflammatory network responses and cell infiltrates.

Cytokines and chemokines as facilitators of a protumor microenvironment

With increased knowledge in endothelial attachment and transcapillary migration, there is now a focus on inflammatory as well as non-inflammatory cell infiltrates and their contribution to cancer cell spread. Chemokines and certain of the larger cytokines may contribute to the migration of leukocytic and other cells into a tumor environment among their other properties. The chemokines now have a new nomenclature based on their chemical structure [28], and extensive reviews have been published [29]. In EOC, particularly in studies on ascites, substantial amounts of certain CC and CXC chemokines have been demonstrated, including CCL18 (PARC), CXCL8 (IL8), CCL2 (MCP1), and CCL3 (MIP1α) [30] (Table 1). Transcripts for CCL4 (MIP1β), CCL5 (RANTES), CCL7 (MCP3) have been demonstrated in EOC cells [31] CCL13, however, is produced by ascitic macrophages and cannot be induced in EOC cells [30]. Chemokines and cytokines may have in common potent functional properties, such as chemotaxis and proangiogenesis, and typically have effects in proximity to cells producing them. Larger cytokine molecules, such as TGFβ, may also have chemotactic and proangiogenic effects. In advanced disease, tumor cells and other cells of nontumor origin, can contribute to chemokine production. CXCL8 (IL8) is very pleiotropic and is constitutively produced or induced by both hematogenous and non-hematogenous cells and by hypoxia. We found that CXCL8 was overexpressed on the peritoneal stroma along with other network genes and appears to be a pivotal chemokine with substantial interactions at the transcript level with genes that are involved in inflammation, angiogenesis, and chemotaxis [27,32]. Receptors for the chemokines are expressed on a variety of hematogenous cells, including T cells and macrophages [33]. Of interest, CXCR4, the receptor for CXCL12 (SDF1), appears to be selectively expressed on EOC cells [34] and may contribute to tumor cell migration. There is a lack of detectable change in expression of other chemokine receptors in response to cytokines, except for CCR2, the receptor for CCL2 and certain other CC chemokines, which appears to be downregulated on EOC ascitic macrophages [35]. This effect may interfere with migration of macrophages away from the tumor site while contributing to a tumor-promoting environment [35]. Unlike cytokines, many chemokines may exhibit more

Table 1: Chemokines/Receptors in EOC

Ligand (Alt. Name)	Receptors	Cells Targeted	Correlates in EOC
CCL2 (MCP1) *+	CCR2	Activated T, Monocytes, DC, Basophils	CD8+ T cells, CD68+ MA ↓ on ascitic MA
CCL3 (MIP1α)*+	CCR2	Activated T, NK, MO, Eosinophils	
CCL4 (MIP1β)*+	Unknown		
CCL5 (RANTES) *	CCR2	Activated T, NK, MO, Eosinophils	
CCL7 (MCP3) +	CCR2		
CCL18 (PARC) +	Unknown		MA produced but not induced in EOC cells
CXCL8 (IL8) *+	CXCR1, CXCR2	Neutrophils, Resting T	
CXCL12 (SDF1) *+	CXCR4	Neutrophils, Resting T, Activated T, B, MO	CXCR4 preferentially expressed on EOC cells

* In RNA detected on EOC cell lines + Proteins detected in ascites

promiscuous binding to receptors. This may insure a regional effect through their redundancy.

Several cytokines have been detected in serum and ascites of EOC patients, including TGFβ isotypes, IL10, IL6, TNFα, CSF1 and IL1 [19,36,37,12,38]. TGFβ isotypes are produced by EOC cells [39] on mononuclear leukocytes, including CD14+DR- [23] and T regulatory cells [22]. TGFβ, in its activated form, was previously considered a tumor-inhibitory cytokine but its tumor-reactive properties appear to be more complex (Table 2). TGFβ also can have a tumor promoting effect in advanced cancer possibly through activation of cdk inhibitors that block the unbinding of the pRb/E2F transcripts [40], and interference with TGFβ receptor binding mediated by H-Ras, as well as consequent to c-myc, its reaction with the E2F transcription factor complex [40]. The signaling pathway of TGFβ within tumor cells may also be subverted due to mutations, or interactions with other cytokines. A TGFβ activation response might, however, prevail in the microenvironment where it may contribute to myofibroblast and endothelial cell chemotaxis, tumor adhesion, and suppression of adaptive and innate immunity [41]. IL10 is also produced in association with EOC with a large contribution by CD14+DR- MO/MA, and these cells may function as immune regulatory cells. IL6 is expressed by EOC tumor cells as well as mesothelial cells and has been detected in the serum and ascites of EOC cells [42-45]. A recent study has shown that IL6 and MCP production by submesothelial cells can be enhanced during abdominal surgery [46]. IL6 also enhances tumor attachment and proliferation of tumor cells, most likely through the PI 3-K activation mechanisms, and can interfere with the maturation of MO/MA to DC [44,45]. This finding might contribute to the large number of functionally immature DC in the ascitic fluid and absent levels of IL12, a product of DC maturation [47,48] (and C Butts' unpublished observations).

Factors associated with compostition and decomposition of the extracellular matrix (ECM)

Phenotypic and functional characterization of stromal inflammatory and non-inflammatory cell infiltrates will be useful for understanding the biology of metastasis. These infiltrates probably occur following transcapillary migration. In this respect, the chemical composition and dynamics of the extracellular matrix (ECM) are also likely to be important. Thus, chemokines may "stick" to other proteins in the stromal microenvironment, enhancing their chemoattraction and other properties by accumulating at these sites. Proteoglycans, which comprise a protein core, and sulphated or non-sulphated aminoglycan side chains could facilitate this. The proteoglycans include a variety of molecules, such as versican, decorin, hyaluran, and heparan with different side chains. The side chain of decorin can be of the dermatin type or chondroitin SO_4 type, each having non-overlapping different functions. We have previously shown that decorin chondroitin SO_4 is expressed with myofibroblasts in the adjacent stroma of EOC tissues [49] (Figure 2). A recent paper has shown that endothelial cells stimulated in culture on a collagen type I matrix in the presence of IL6 and IL10 synthesized decorin [50]. This is of particular interest since both IL6 and IL10 are highly expressed in EOC. Chemokines may attach covalently to proteoglycans that express GAG sequences, while retaining their effects on tumor microenvironment cells. This may facilitate their effects locally. In contrast, proteoglycans might also interfere with the binding of activated TGFβ to its receptors.

A large family of receptors called integrins can regulate many functions at both the cellular and ECM levels. The integrins are important for the spread and proliferation of cancer cells [2]. Their functions, however, are complex since integrins can associate with other integrins or growth factor receptors or adaptive proteins producing bidirectional effects to and from the cell membrane surface. Integrin mediated effects include cytoskeletal changes through complexing with α actinin and other proteins with downstream effects on actin. These changes may

Table 2: Dual Effects of Cytokines on Tumor/Tumor Microenvironment

TGFβ	IL6
• TGFβ + TGFβ RIII → TGFβ - RII + RI heterodimer → TGFβ RI - P + SMADs → SMAD - P → nucleus → initiates transcription • TGFβ → in repression cell cycle genes or activation • Repression involves activation of cycle dependent kinase inhibitors, blocks unbinding of pRb/E2F transcripts • Other interactions include: H-Ras (↓ RI & ↑ RII); C-Myc (stimulates proliferation by repressing cdk inhibitors) associates w/E2F transcript factor complex • TGFβ effect negated by disruption of signal pathway • Alterations to the microenviroment Tumor adhesion Endothelial chemotaxis (proangiogenic) Myofibroblast chemotaxis • Immunosuppressive Effects Adaptive ↓ MHC expression (targeting) ↓ Costimulatory Ag expression by DC Blocks pre CTL → CTL Suppresses TH1 cells - Shift to TH2 Induces apoptosis Suppresses proliferative response to APCs Innate Cells Inhibits NK & MA activation	• ↑ TuC attachment migration • Immune modulation (T-cell ↑) • Interferes w/MA maturation to DC • Proliferation thru PI3-K activation **IL10** • ↓ MHC expression on TC • ↓ Costimulatory Ag expression • Suppress cytotoxic T-cell activation • Inhibits IFNγ production • Inhibits T-cell production

affect cell survival, proliferation, motility, and differentiation. Depending upon the particular signaling pathway dominance, the downstream effect may be either repression or activation of a particular function. Integrins may modulate or enhance the expression of other integrins and receptors, e.g. β1 and β3 integrins, which can influence apoptosis. Lack of detection of the cell-to-cell adhesion molecule E-cadherin on cuboidal cells of ovarian surface epithelium in contrast to its upregulated expression on metaplastic intra-ovarian cystic glands and early well-differentiated glandular carcinoma suggest that the latter cells may have been derived from the migrating surface ovarian cells [51]. E-cadherin also inhibits the anti-apoptotic PI3K signaling pathway and E-cadherin expression in advanced EOC metastatic nodules appears to be less prominent than other cadherin molecules [52]. In a 3-D model of breast cancer, chronic activation of the β1 integrin has been shown to enhance the cancer phenotype in contrast to a different signaling effect from α6/β4 integrin activation, leading to suppression of the cancer phenotype. In advanced EOC, the reduction or loss of E-cadherin expression is hypothesized to contribute to the spread and progression of the tumor [52]. E-cadherin in both breast and ovarian cancers is considered a late tumor suppressor molecule. The importance of β1 signaling has been shown in experiments that demonstrate reversion of the malignant phenotype when β1 integrin is blocked with anti β1 mAbs [53]. These findings overall suggest that altered expression of adhesion molecules, such as cadherein, could serve different functions during the

pathogenesis of the EOC disease process. Antibody-mediated inhibition of integrin 1 has also been shown to interefere with production of decorin which is an important part of the ECM [50].

Animal models for EOC/peritoneal interactions
Representative animal models of EOC need to combine the oncogenic developmental pathways as well as contributions from the cellular and ECM environment of the epithelium and stroma. This relationship, however, is clearer in established virally induced tumors [4].

There has been some recent progress in the development of suitable mouse models for human ovarian cancer [54,55]. Because of marked heterogeneity of ovarian cancer both at histopathologic and clinical levels, the underlying mechanisms that produce the altered gene expression profile in the EOC is not clear. However, it is reasonable to assume that the altered gene expression profile is at least in part due to activation of oncogenic events that transform the ovarian surface epithelial cells. Toward this end, it has been shown that oncogenic HRAS[V12] or KRAS[V12] activates multiple proinflammtory cytokines and angiogenic factor cytokines during the malignant transformation of ovarian surface epithelial cells in a newly created genetically defined model for ovarian cancer. In this model, introduction of SV40 T/t antigen extended the life span of primary cultured ovarian surface epithelial cells for a few more passages; however, these T/t antigen-expressing cells are still mortal. Introduction of the cata-
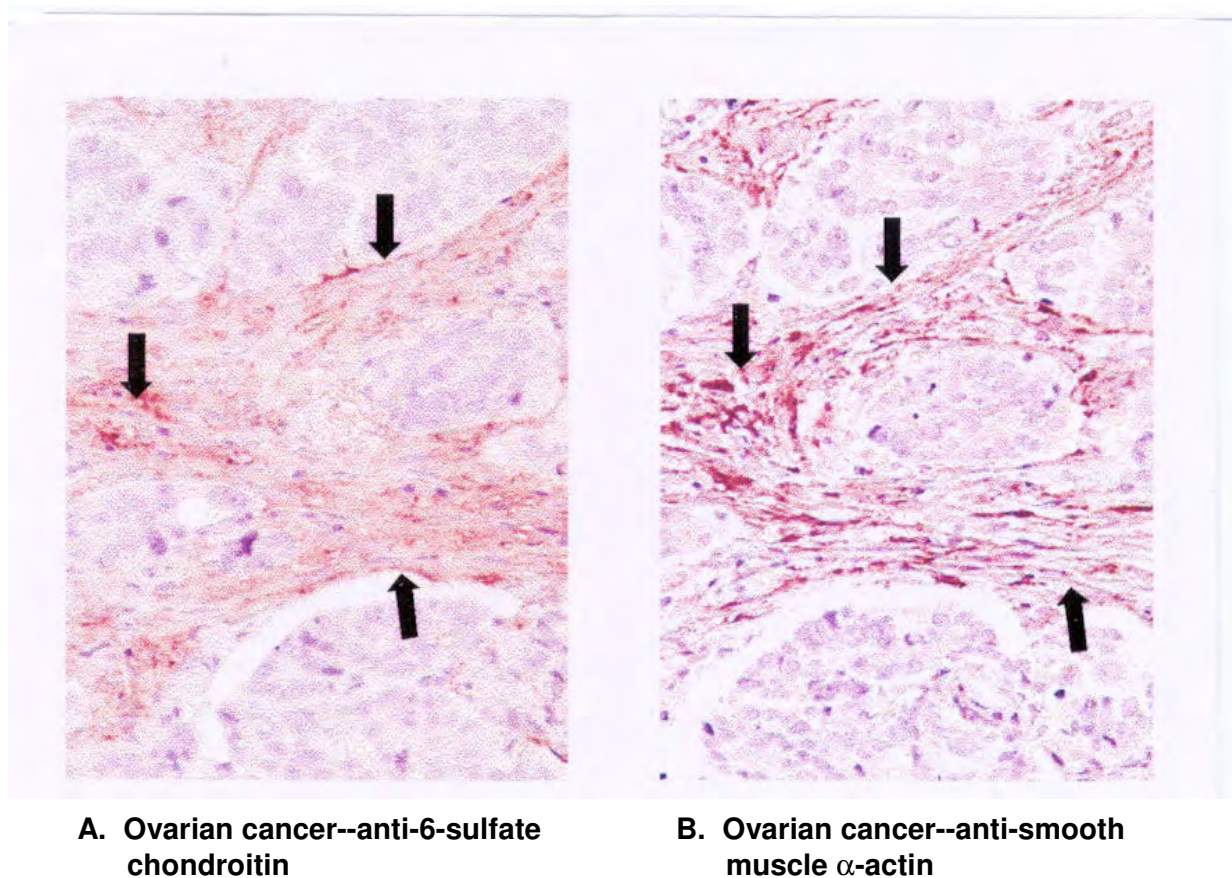
A. Ovarian cancer--anti-6-sulfate chondroitin

B. Ovarian cancer--anti-smooth muscle α-actin

Figure 2
Histochemical staining of human ovarian tissue with anti-6-sulfate chondroitin or anti-smooth muscle α-actin antibody. Serial 5-μm sections of paraffin-embedded tumors were stainead with anti-6-sulfate chondroitin (A) or anti-smooth muscle α-actin (B). *Arrows* point to regions of staining overlap. (Re-printed with permission from *Clinical Cancer Research*).

lytic subunit of telomerase (hTERT) results in immortalization of these cells. Introduction of HRAS[V12] or KRAS[V12] results in transformation of these cells as reflected on the increased number of anchorage independent growth and tumor development after subcutaneous injection of these cells. Peritoneal injection of the transformed cells produced undifferentiated carcinoma or malignant mixed mullerian tumor and developed ascites, the tumor cells are focally positive for CA125 and mesothelin. Gene expression profile analysis of transformed cells revealed elevated expression of several cytokines including interleukin (IL)-1β, IL6, and IL8, that are up-regulated by the NF-κB pathway, which is known to contribute to naturally occurring human EOC. Incubation with antibodies to IL-1β or IL8 led to apoptosis in the ras-transformed cells and ovarian cancer cells but not in immortalized cells that had not been transformed. Thus, the transformed human ovarian surface epithelial cells recapitulated many features of natural ovarian cancer including a subtype of ovarian cancer histology, formation of ascites, CA125 expression, and NF-κB-mediated cytokine activation. These cells provide a novel model system to study human ovarian cancer. Because of the remarkable similarity of gene expression between the RAS-transformed ovarian surface epithelial cells and peritoneum associated with ovarian cancer, these immortalized preneoplastic ovarian epithelial cell lines may provide a valuable experimental tool to examine the role of each of the cytokines in the peritoneum during ovarian cancer development [54].

Susceptibility of the stromal compartment of the peritoneum to proliferative signals has been well documented in animal models of the peritoneal fibrosing syndrome following exposure to chemical peritoneal dialysates [9,56]. In these models, alterations to the stromal environment occur in response to the dialysate resulting in infiltration of two main cell populations, fibroblasts secreting MCP-1, VEGF, and HSP47 and macrophages which can express TGFβ, TNFα, IL1 and fibronectin. Macrophages in these models were shown to be recruited by several CC chemokines, MCP-1, RANTES, and MIP-1 and collagen-dependent endothelial cells. Moreover, removal of the fibroblast element in a knockout animal model abrogated the MA infiltration and the fibrotic process. A similar process has been described in renal fibrosis, which also involves mononuclear leukocytes and myofibroblasts. In EOC, it is possible that molecules derived from the primary or peritoneal surface metastases could be distributed throughout the peritoneal cavity. Even in the absence of ascites, the distribution of these molecules could possibly be facilitated by negative pressure in the peritoneal cavity and peristalsis of the intestines. The precise mechanisms underlying the formation of ascites is unknown. Ascites indicate a more advanced stage of the disease which could be a consequence of alterations in permeability of the peritoneum or extensive lymphatic obstruction. In subperitoneal metastatic growth, the ascites may be almost acellular whereas surface exophytic lesions may be accompanied by large numbers of free-floating tumor cells, mesothelial cells and leukocytes, and in some cases, the ascites has a hemorrhagic appearance.

In summary, we have shown that peritoneal structures of patients with EOC are different at the transcript level from those of patients with benign conditions [27]. The changes observed reflect alterations in cytoskeletal and signaling pathways that suggest regional activity from integrins, cytokines, hormone growth factors, and adaptive proteins. In addition, there appears to be intense chemokine activity, particularly of the CXC motif chemokines, suggesting a pattern of chemotactic influence on leukocytic as well as other cell types. Enhanced collagenase activity would contribute to remodeling of the stromal compartment and creation of a favorable environment for infiltration of leukocytes as well as other cells, such as myofibroblasts and endothelial cells. Gene profiling of the peritoneum may provide hints about early transition steps to cancer or provide insight into changes that may actually facilitate the spread of cancer to adjoining tissues. It is anticipated that future studies using high throughput technologies with a multidimensional approach will enhance understanding of these alterations and their biological significance. These efforts could help identify critical alterations in the environment surrounding the cancer and its metastases and might ultimately lead to advances in diagnosis, prognosis, and novel approaches to therapeutic targeting in EOC.

The past few decades have seen considerable progress in chemotherapeutics of EOC that are contributing to an overall reduction in mortality [57]. However, EOC is heterogenous in its histopathology and sensitivity to chemotherapy. In order to overcome redundancies in the pathways and networks that control tumor cell growth, it will be necessary to employ multitargeted therapeutic strategies. A number of peritoneal structures could serve as useful potential targets, including inflammatory and non-inflammatory stromal cells, as well as production of molecules in the ECM, such as chemokines [29]. *In vitro* experiments suggest that the microenvironment can influence the malignant phenotype. It is also likely that malignant cells from the primary tumor or metastasis might modify the microenvironment, preparing both surface epithelial and stromal cells to support the growth and proliferative activity of the tumor. Thus, future strategies should attempt to identify those pathways and networks in the microenvironment that are critical to tumor cell survival.

References

1. Rubin SC, Randall TC, Armstrong KA, Chi DS, Hoskins WJ: **Ten-year follow-up of ovarian cancer patients after second-look laparotomy with negative findings.** *Obstetrics & Gynecology* 1999, **93**:21-24.
2. Miranti CK, Brugge JS: **Sensing the environment: a historial perspective on integrin signal transduction.** *Nature Cell Biol* 2002, **4**:83-90.
3. Bissell MJ, Radisky DC, Rizki A, Weaver VM, Petersen OW: **The organizing principle: microenvironmental influences in the normal and malignant breast.** *Differentiation* 2002, **70**:537-546.
4. Coussens LM, Werb Z: **Inflammation and cancer.** *Nature* 2002, **420**:860-867.
5. Tuhkanen H, Anttila M, Kosma V-M, Yla-Herttuala S, Heinonen S, Kuronen A, Juhola M, Tammi R, Tammi M, Mannermaa A: **Genetic alterations in the peritumoral stromal cells of malignant and borderline epithelial ovarian tumors as indicated by allelic imbalance on chromosome 3P.** *Int J Cancer* 2004, **109**:247-252.
6. Carter D, True L, Otis CN: **Serous Membranes.** *Histology for Pathologists, 2nd Edition* Edited by: Sternberg SS. Philadelphia, Lippincott-Raven; 1997:223-239.
7. Battifora H, McCaughey WTE: **Tumors of the serosal membranes.** *Atlas of tumor pathology Third series* Edited by: Universities Associated for Research and Education in Pathology Inc. Bethesda, MD, Armed Forces Institute of Pathology; 1995.
8. Eddy AA: **Molecular basis of renal fibrosis.** *Pediatr Nephrol* 2000, **15**:290-301.
9. Okada H, Inoue T, Kanno Y, Kobayashi T, Watanabe Y, Ban S, Neilson EG, Suzuki H: **Selective depletion of fibroblasts preserves morphology and the functional integrity of peritoneum in transgenic mice with peritoneal fibrosing syndrome.** *Kidney International* 2003, **64**:1722-1732.
10. Dedrick RL, Flessner MF: **Pharmacokinetic problems in peritoneal drug administration: tissue penetration and surface exposure.** *J Natl Cancer Inst* 1997, **89**:480-487.
11. Freedman RS, Kudelka AP, Kavanagh JJ, Verschraegen C, Edwards CL, Nash M, Levy L, Atkinson EN, Zhang HZ, Melichar B, Patenia R, Templin S, Scott W, Platsoucas CD: **Clinical and biological effects of intraperitoneal injections of recombinant interferon-g, and recombinant interleukin-2 with or without tumor-infiltrating lymphocytes in patients with ovarian or peritoneal carcinoma.** *Clin Cancer Res* 2000, **6**:2268-2278.

http://www.translational-medicine.com/content/2/1/23

12. Freedman RS, Edwards CL, Kavanagh JJ, Kudelka AP, Katz RL, Carrasco CH, Atkinson EH, Scott W, Tomasovic B, Platsoucas CD: **Intraperitoneal adoptive immunotherapy of ovarian carcinoma with tumor infiltrating lymphocytes, A pilot trial.** *J Immunother Emphasis Tumor Immunol* 1994, **16:**198-210.

13. Ioannides CG, Freedman RS, Platsoucas CD, Rasheed S, Kim YP: **Cytotoxic T cell clones isolated from ovarian tumor-infiltrating lymphocytes recognize multiple antigenic epitopes on autologous tumor cells.** *J Immunol* 1991, **146:**1700-1707.

14. Freedman RS, Tomasovic B, Templin S, Atkinson EN, Kudelka A, Edwards CL, Platsoucas CD: **Large-scale expansion in interleukin-2 of tumor-infiltrating lymphocytes from patients with ovarian carcinoma for adoptive immunotherapy.** *J Immunol Methods* 1994, **167:**145-160.

15. Yoshino I, Peoples GE, Goedegebuure PS, Maziarz R, Eberlein TJ: **Association of HER2/neu expression with sensitivity to tumor specific CTL in human ovarian cancer.** *J Immunol* 1994, **152:**2393-2400.

16. Disis ML, Smith JW, Murphy AE, Chen W, Cheever MA: **In vitro generation of human cytolytic T-cells specific for peptides derived from the HER-2/neu proto-oncogene protein.** *Cancer Research* 1994, **54:**1071-1076.

17. Zhang Lin, Conejo-Garcia JR, Katsaros D, Gimotty PA, Massobrio M, Regnani G, Makrigiannakis A, Gray H, Schlienger K, Liebman MN, Rubin S, Coukos George: **Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer.** *N Engl J Med* 2003, **348:**203-213.

18. Pappas J, Lin WL, Helm CW, Freedman RS, Platsoucas CD: **Clonally expanded T cell receptor (TCR) transcripts in ovarian tumor infiltrating lymphocytes (TIL).** *Scand J Immunol (supplement)* 2001, **54:**84.

19. Nash MA, Lenzi R, Edwards CL, Kavanagh JJ, Kudelka AP, Verschraegen CF, Platsoucas CD, Freedman RS: **Differential expression of cytokine transcripts in human epithelial ovarian carcinoma by solid tumor specimens, peritoneal exudate cells containing tumor, TIL-derived T-cell lines and established tumor cell lines.** *Clin Exp Immunol* 1998, **112:**172-180.

20. Rabinowich H, Suminami Y, Reichert TE, Crowley-Nowick P, Bell M, Edwards R, Whiteside TL: **Expression of cytokine genes or proteins and signaling molecules in lymphocytes associated with human ovarian carcinoma.** *Int J Cancer* 1996, **68:**276-284.

21. Lenzi R, Rosenblum M, Verschraegen C, Kudelka AP, Kavanagh JJ, Hicks ME, Lang EA, Nash MA, Levy LB, Garcia ME, Platsoucas CD, Abbruzzese JL, Freedman RS: **Phase I study of intraperitoneal rhIL-12 in patients with mullerian carcinoma, gastrointestinal primary malignancies and mesothelioma.** *Clinical Cancer Research* 2002, **8:**3686-3695.

22. Woo Edward Y, Chu Christina S, Goletz Theresa J, Schlienger K, Yeh Heidi, Coukos George, Rubin Stephen C., Kaiser Larry R, June Carl H: **Regulatory CD4+CD25+ T cells in tumors from patients with early-stage non-small cell lung cancer and late-stage ovarian cancer.** *Cancer Res* 2001, **61:**4766-4772.

23. Loercher AE, Nash MA, Kavanagh JJ, Platsoucas CD, Freedman RS: **Identification of an IL-10 producing HLA-DR-negative monocyte subset in the malignant ascites of patients with ovarian carcinoma that inhibits cytokine protein expression and proliferation of autologous T cells.** *J Immunol* 1999, **163:**6251-6260.

24. Mantovani A, Sozzani S, Locati Massimo, Allavena P, Sica A: **Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes.** *Trends in Immunology* 2002, **23:**549-555.

25. Mocellin S, Panelli MC, Wang E, Nagorsen D, Marincola FM: **The dual role of IL-10.** *Trends in Immunology* 2003, **24:**36-43.

26. Okrent IA, Freedman RS: **Monocyte and macrophage (MO/MA) subpopulations in epithelial ovarian cancer (EOC.** *Proc of AACR* 2004, **45:**1259a.

27. Wang E, Ngalame Y, Panelli MC, Nguyen-Jackson H, Deavers Michael, Mueller P, Hu W, Savary C, Freedman RS, Marincola FM: **Peritoneal and sub-peritoneal stroma may facilitate regional spread of ovarian cancer.** *Proc of AACR* 2004, **45:**1778a.

28. IUIS/WHO Subcommittee: **Chemokine/chemokine receptor nomenclature.** *Cytokine* 2003, **21:**48-49.

29. Oppenheim JJ, Howard OMZ, Goetzl E: **Chemotactic factors, neuropeptides, and other ligands for seven transmembrane receptors.** *Cytokine Reference Volume 1:Ligands.* Edited by: Oppen-

heim JJ and Feldmann. San Diego, CA, Academic Press; 2001:985-1021.

30. Schutyser E, Struyf S, Proost P, Opdenakker G, Laureys G, Verhasselt B, Peperstraete L, Van de Putte I, Saccani A, Allavena P, Mantovani A, Van Damme J: **Identification of biologically active chemokine isoforms from ascitic fluid and elevated levels of CCL18/pulmonary and activation-regulated chemokine in ovarian carcinoma.** *J of Biol Chem* 2002, **277:**24584-24593.

31. Negus RP, Stamp GW, Relf MG, Burke F, Malik ST, Bernasconi S, Allavena P, Sozzani S, Mantovani A, Balkwill FR: **The detection and localization of monocyte chemoattractant protein-1 (MCP-1) in human ovarian cancer.** *Journal of Clinical Investigation* 1995, **95:**2391-2396.

32. Negus RP, Stamp GW, Hadley J, Balkwill FR: **Quantitative assessment of the leukocyte infiltrate in ovarian cancer and its relationship to the expression of C-C chemokines.** *Am J Pathology* 1997, **150:**1723-1734.

33. Scotton Chris J., Wilson Julia L., Scott Kate A., Stamp Gordon, Wilbanks George D, Fricker Simon, Bridger Gary, Balkwill FR: **Multiple actions of the chemokine CXCL12 on epithelial tumor cells in human ovarian cancer.** *Ca Research* 2002, **62:**.

34. Sica A, Saccani A, Mantovani Alberto: **Tumor-associated macrophages: a molecular perspective.** *International Immunopharmacology* 2002, **2:**1045-1054.

35. Moradi Mark M, Carson LF, Weinberg JB, Haney AF, Twiggs Leo B, Ramakrishnan S: **Serum and ascitic fluid levels of interleukin-1, interleukin-6, and tumor necrosis factor-alpha in patients with ovarian epithelial cancer.** *Cancer* 1993, **72:**2433-2440.

36. Burke Frances, Relf Michele, Negus RP, Balkwill FR: **A cytokine profile of normal and malignant ovary.** *Cytokine* 1996, **8:**578-585.

37. Berek JS, Chung C, Kaldi K, Watson JM, Knox RM, Martinez-Maza O: **Serum interleukin-6 levels correlate with disease status in patients with epithelial ovarian cancer.** *Am J Obstet Gynecol* 1991, **164:**1038-1042.

38. Gordinier ME, Zhang HZ, Patenia R, Levy LB, Atkinson EN, Nash MA, Katz RL, Platsoucas CD, Freedman RS: **Quantitative analysis of transforming growth factor-beta (TGF-b1 and TGF-b2) on ovarian carcinoma.** *Clin Cancer Res* 1999, **5:**2498-2505.

39. Chen Chang-Rung, Kang Yibin, Siegel Peter M, Massague Joan: **E2F4/5 and p107 as Smad cofactors linking the TGFbeta receptor to c-myc repression.** *Cell* 2002, **110:**.

40. Nash MA, Ferrandina G, Gordinier M, Loercher A, Freedman RS: **The role of cytokines in both the normal and malignant ovary.** *Endocrine Related Cancer* 1999, **6:**93-107.

41. Berek JS, Welander C, Schink JC, Grossberg H, Montz FJ, Zigelboim J: **A phase I-II trial of intraperitoneal cisplatin and a-interferon in patients with persistent epithelial ovarian cancer.** *Gynecol Oncol* 1991, **40:**237-243.

42. Offner FA, Obrist P, Stadlmann S, Feichtinger H, Klingler P, Herold M, Zwierzina HH, Hittmair A, Mikuz G, Abendstein B: **IL-6 secretion by human peritoneal mesothelial and ovarian cancer cells.** *Cytokine* 1995, **7:**542-547.

43. Obata NH, Tamakoshi K, Shibata K, Kikkawa F, Tomoda Y: **Effects of interleukin-6 on in vitro cell attachment, migration and invasion of human carcinoma.** *Anticancer Research* 1997, **17:**337-342.

44. Tempfer C, Zeisler H, Sliutz G, Haeusler G, Hanzal E, Kainz C: **Serum evaluation of interleukin 6 in ovarian cancer patients.** *Gynecol Oncol* 1997, **66:**27-30.

45. Riese J, Niedobitek G, Lisner R, Jung A, Hohenberger W, Haupt W: **Expression of interleukin-6 and monocyte chemoattractant protein-1 by peritoneal sub-mesothelial cells during abdominal operations.** *J Pathology* 2004, **202:**34-40.

46. Melichar B, Savary C, Kudelka AP, Verschraegen C, Kavanagh JJ, Edwards CL, Platsoucas CD, Freedman RS: **Lineage-negative human leukocyte antigen-DR+ cells with the phenotype of undifferentiated dendritic cells in patients with carcinoma of the abdomen and pelvis.** *Clin Cancer Res* 1998, **4:**799-809.

47. Freedman RS, Vadhan-Raj S, Butts C, Savary C, Melichar B, Verschraegen C, Kavanagh JJ, Hicks ME, Levy LB, Folloder J, Garcia ME: **Pilot study of Flt3 ligand comparing intraperitoneal with subcutaneous routes on hematologic and immunologic responses in patients with peritoneal carcinomatosis and mesotheliomas.** *Clin Cancer Res* 2003, **9:**5228-5237.

http://www.translational-medicine.com/content/2/1/23

48. Nash MA, Deavers MT, Freedman RS: **The expression of decorin in human ovarian tumors.** *Clinical Cancer Research* 2002, **8**:1754-1760.
49. Strazynski Marco, Eble Johannes A., Kresse Hans, Schonherr Elke: **Interleukin (IL)-6 and IL-10 induce decorin mRNA in endothelial cells, but interaction with fibrillar collagen is essential for its translation.** *J of Biol Chem* 2004, **279**:21266-21270.
50. Roskelley CD, Bissell MJ: **The dominance of the microenvironment in breast and ovarian cancer.** *Cancer Biology* 2002, **12**:97-104.
51. Patel IS, Madan P, Getsios S, Bertrand MA, MacCalman CD: **Cadherin switching in ovarian cancer progression.** *Int J Cancer* 2003, **106**:.
52. Weaver VM, Petersen OW, Wang F, Larabell CA, Briand P, Damsky C, Bissell MJ: **Reversion of the Malignant Phenotype of Human Breast Cells in Three-Dimensional Culture and In Vivo by Integrin Blocking Antibodies.** *J Cell Biol* 1997, **137**:231-245.
53. Liu Jinsong, Yang Gong, Thompson-Lanza Jennifer A, Glassman Armand, Hayes Kimberly, Patterson Andrea, Marquez Rebecca T, Auersperg Nelly, Yu Yinhua, Hahn William C, Mills Gordon B, Bast RC: **A genetically defined model for human ovarian cancer.** *Cancer Res* 2004, **64**:1655-1663.
54. Orsulie S, Li Y, Soslow RA, Vitale-Cross LA, Gutkind JS, Varmus HE: **Induction of ovarian cancer by defined multiple genetic change in a mouse model system.** *Cancer Cell* 2002, **1**:53-62.
55. Dobbie J: **Pathogenesis of peritoneal fibrosing syndromes (sclerosing paeritonitis) in peritoneal dialysis.** *Perit Dial Int* 1992, **12**:14-27.
56. Jemal Ahmedin, Clegg Limin, Ward Elizabeth, Ries LAG, Wu Xiaocheng, Jamison Patricia, Wingo Phyllis, Howe Holly, Anderson Robert, Edwards Brenda K: **Annual Report to the Nation on the Status of Cancer, 1975-2001, with a Special Feature Regarding Survival.** American Cancer Society; 2004:1-25.
57. Jemal Ahmedin, Clegg Limin, Ward Elizabeth, Ries LAG, Wu Xiaocheng, Jamison Patricia, Wingo Phyllis, Howe Holly, Anderson Robert, Edwards Brenda K: **Annual Report to the Nation on the Status of Cancer, 1975-2001, with a Special Feature Regarding Survival.** American Cancer Society; 2004:1-25.

Exhibit 83

Modulation of redox signaling promotes apoptosis in epithelial ovarian cancer cells

Zhongliang Jiang[a], **Nicole M. Fletcher**[a], **Rouba Ali-Fehmi**[b], **Michael P. Diamond**[a], **Husam M. Abu-Soud**[a], **Adnan R. Munkarah**[c], and **Ghassan M. Saed**[a,*]

[a] Department of Obstetrics and Gynecology, The C.S. Mott Center for Human Growth and Development, Wayne State University School of Medicine, Detroit, MI, USA

[b] Department of Pathology, Wayne State University, USA

[c] Department of Women's Health Services, Henry Ford Health System, USA

Abstract

Objective—Epithelial ovarian cancer (EOC) cells are known to be resistant to apoptosis through a mechanism that may involve alteration in their redox balance. NADPH oxidase is a major source of intracellular superoxide, which is converted to the less toxic product by superoxide dismutase (SOD). Superoxide contributes to hypoxia inducible factor (HIF)-1α stabilization. We sought to determine the effects of inhibiting the generation of intracellular reactive oxygen species (ROS) on apoptosis of EOC cells.

Methods—Diphenyleneiodonium (DPI), an irreversible ROS inhibitor, was used to inhibit the generation of ROS in EOC cell lines, SKOV-3 and MDAH-2774, followed by assessment of apoptosis, NADPH oxidase, SOD3 and HIF-1α expression. A combination of immunohistochemistry, immunoprecipitation/western blot, and real-time RT-PCR were utilized to evaluate the expression of these enzymes in EOC cells as well as normal ovarian tissue and ovarian cancer tissue specimens.

Results—DPI treatment significantly induced apoptosis in both EOC cell lines as evident by increased caspase-3 activity and TUNEL assay. Additionally, both EOC cell lines were found to express NADPH oxidase, HIF-1α, and SOD3, which were highly sensitive to DPI treatment. DPI treatment resulted in reduced NADPH oxidase, SOD3 and HIF-1α levels. Furthermore, ovarian

*Corresponding author at: Department of Obstetrics & Gynecology, C. S. Mott Center for Human Growth and Development, Wayne State University School of Medicine, 275 E. Hancock, Detroit, MI 48201. Fax: +1 313 577 4633. gsaed@med.wayne.edu (G.M. Saed)..

Conflict of interest statement
Zhongliang Jiang – None
Nicole M. Fletcher – None
Rouba Ali-Fehmi – None
Michael P. Diamond – Consultant for the following: Omrix, Genzyme, Neomend, Baxter, EMD Serono, Snaofi-Aventis, Half Medical, ZSX Medical, and Auzogyn. Board Member for the following: Advanced Reproductive Care, American Society for Reproductive Medicine. Research Grants for the following: Boehringher Ingelheim BirSante, Johnson and Johnson, Interlace Medical, Glaxo-Smith Klein, Neurocrine Biosciences. Stocks for the following: Neomend. Speaker Bureau: Boehringher Ingelheim.
Husam M. Abu-Soud – None
Adnan R. Munkarah – None
Ghassan M. Saed – None

cancer tissues were found to manifest higher NADPH oxidase levels as compared to normal ovarian tissues.

Conclusions—These data suggest that lowering oxidative stress, possibly through the inhibition of NADPH oxidase, induces apoptosis in ovarian cancer cells and may serve as a potential target for cancer therapy.

Keywords

NADPH oxidase; Diphenyleneiodonium; Epithelial ovarian cancer; Oxidative stress; Superoxide dismutase; Hypoxia inducible factor-1 alpha

Introduction

Ovarian cancer is the fifth leading cause of cancer death in women, the leading cause of death from gynecologic malignancies, and the second most commonly diagnosed gynecologic malignancy, however the underlying pathophysiology is not clearly understood [1]. Malignant cells are resistant to apoptosis through a mechanism that may involve alterations in their redox balance. Reactive oxygen species (ROS), generated in the mitochondria as a byproduct of oxidative phosphorylation, are continuously generated and eliminated in the biological system and play important roles in a variety of normal biochemical functions and abnormal pathological processes [2–5].

In addition to the ROS produced by the mitochondria, nicotin-amide adenine dinucleotide phosphate (NADPH)-oxidase, a flavoenzyme family member, generates a significant amount of endogenous ROS through the reduction of O_2 to superoxide $\left(O_2^{\cdot-}\right)$, hydrogen peroxide ($H_2O_2$), and other ROS [6–9]. The NADPH oxidase complex is composed of multiple subunits, including a glycoprotein gp91-phox, which is considered to be directly involved in the generation of $O_2^{\cdot-}$ [8]. Enzyme systems similar to the phagocyte NADPH oxidase are now known to exist in many other cell types, and are referred to as the NOX family of NADPH oxidases [8–13].

Several antioxidant enzymes, including superoxide dismutases (SOD), catalase, and various peroxidases are effective in removing destructive ROS [14,15]. Superoxide dismutases are key enzymes required for removal of $O_2^{\cdot-}$, thorough its conversion to H_2O_2, which is further eliminated by both catalase and peroxidases [15,16]. Human extracellular Cu/Zn SOD (SOD3), is a unique SOD family member found in the extracellular matrix of tissues and is ideally situated to prevent cell and tissue damage, initiated by extracellularily produced ROS [17]. The loss of endogenous SOD3 activity can exacerbate oxidative stress and pathologic damage as it is a critical endogenous antioxidant enzyme involved in carcinogenesis, cancer proliferation and metastasis [18].

Cancer cells are under intrinsic oxidative stress and manifest significantly increased levels of ROS, and thus express higher levels of SOD, which has been reported to stabilize the HIF-1α protein, enabling HIF-1α to dimerize, forming an active transcription factor [11,19–21]. Since rapidly growing tumors become hypoxic, questions are raised as to what promotes an increase in SOD, and why HIF-1α is stabilized and not degraded, with the

Jiang et al.   Page 3

increase in ROS under the hypoxic environment in cancer cells [22,23]. Restoration of the ROS balance in cancer cells may provide a potential therapeutic intervention to selectively eliminate cancer cells via apoptosis. Inhibition of NADPH oxidase and other pro-oxidant enzymes has been reported to significantly induce apoptosis of cancer cells [24,25]. Several agents have been utilized to test this hypothesis, however, few have been tested in ovarian cancer [26–28].

In the present study, we investigated whether NADPH oxidase-mediated generation of intracellular reactive ROS lead to anti-apoptotic activity and thus a growth advantage to epithelial ovarian cancer (EOC) cells. Specifically, we have utilized DPI, an irreversible inhibitor of flavoproteins, including NADPH oxidase, to evaluate apoptosis of EOC cells. Additionally, we have evaluated other key players in the regulation of redox homeostasis and apoptosis, including SOD3 and HIF-1α. Identification of targets to specifically induce apoptosis in EOC cells may provide a potential therapeutic target for selective elimination of cancer cells.

Materials and methods

Culture of EOC cell lines

The two human EOC cell lines, SKOV-3 and MDAH-2774, were obtained from ATCC (ATCC, Manassas, VA). Cells were cultured in $75cm^2$ cell culture flasks (Corning Incorporated, Corning, NY) with McCoy's 5A medium (Invitrogen, Carlsbad, CA) supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin including 10% heat-inactivated FBS at 37 °C in 5% CO_2. Culture medium was replaced every two days.

Treatment of human EOC cells

Cells were plated (5×10^6) in 100 cm^2 culture dishes and incubated for 24 hours. Cell were treated with 10 μM DPI (Sigma-Aldrich, Saint Louis, MO) for 0, 0.5, 1, 3, 6, 12, and 24 hours. The dose was selected based on previous studies [26–28]. Cells were harvested at each time point. All experiments were performed in triplicate.

Measurement of caspase-3 activity in EOC cells

A Caspase-3 Colorimetric Activity Assay Kit (Chemicon, Billerica, MA) was utilized per the manufacturer's protocol. Cells (2×10^6) were harvested and lysed in 300 μl of lysis buffer, and concentrations were equalized for each sample set. Cell lysate (150 μg) was combined with substrate reaction buffer containing 30 μg of caspase-3 substrate, acetyl-DEVD-p-nitroaniline (Ac-DEVD-pNA). This mixture was incubated for 1 hr at 37 °C, and then absorbance was measured with a plate reader (Ultramark, BIO-RAD, Hercules, CA).

Detection of apoptosis in EOC cells

DNA fragmentation was assessed by the in situ Terminal Deoxynucleotidyl Transferase-mediated dUTP-biotin nick end labeling (TUNEL) technique per the DeadEnd Fluorometric TUNEL System (Promega, Madison, WI) manufacturer's protocol. Briefly, the cells were fixed with 4% paraformaldehyde in PBS for 20 min at 4 °C, and subjected to permeabilization for 5 min at room temperature with 0.2% Triton X-100. Next, cells were

Jiang et al.   Page 4

labeled with the TUNEL reaction mixture for 60 min at 37 °C. The nuclei of these cells were also stained with 4′,6′-diamino-2-phenylindole (DAPI). Fluorescein-labeled DNA, an indication of DNA fragmentation, was analyzed by using an Axiovert 40 CFL immuno-fluorescent microscope (Carl Zeiss Microimaging, Thornwood, NY) and recorded with a microscope-mounted camera (Carl Zeiss).

Real-time reverse transcription polymerase chain reaction (RT-PCR) for and HIF-1α, NADPH oxidase, and SOD3

RNA isolation—Total RNA was extracted from both SKOV-3 and MDAH-2774 cells using an RNeasy Mini Kit (QIAGEN, Valencia, CA) according to the protocol provided by the manufacturer.

Reverse transcription—A 20 μL cDNA reaction volume was prepared with the use of QuantiTect Reverse Transcription Kit (QIAGEN) according to the protocol provided by the manufacturer.

Real-time RT-PCR—Real-time RT-PCR was performed with a QuantiTect SYBR Green RT-PCR kit (QIAGEN) and a Cepheid 1.2f Detection System (Cepheid, Sunnyvale, CA). Each 25-μL reaction included 12.5 μL of 2×QuantiTect SYBR Green RT-PCR master mixes, 1 μL of cDNA template, and 0.2 μM each of target-specific primer were selected with the aid of the software program, Beacon Designer (Premier Biosoft, Palo Alto, CA). Human oligonucleotide primers (HIF-1α, p22-phox, and SOD3) that amplify variable portions of the protein coding regions are listed in Table 1. Standards with known concentrations and lengths (base pairs (bp)) were designed specifically for HIF-1α (100 bp), NADPH oxidase p22-phox subunit (82 bp), and SOD3 (99 bp) using the Beacon Designer software (Premier Biosoft), allowing for construction of a standard curve using a tenfold dilution series. A specific standard for each gene allows for absolute quantification of the gene in number of copies, which can then be expressed per μg of RNA. The conditions for the three-step polymerase chain reaction protocol were as follows: an initial cycle at 95 °C for 900 s (HIF-1α), 1000 s (p22-*phox*) and 850 s (SOD3) followed by 35 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C (HIF-1α and SOD3) and 54 °C (p22-*phox*) for 30 s, and extension at 72 °C for 30 s. Finally, a melting curve analysis was performed to demonstrate the specificity of the PCR product as a single peak. A control, which contained all the reaction components except for the template, was included in all experiments.

Measurement of SOD3 protein levels in EOC cells

Immunoprecipitation (IP)/Western blot was utilized as previously described with the following changes [29]. Cells were lysed with lysis buffer and cleared by centrifugation (10 minutes at 1,000 g, 4 °C). Protein concentration of cell lysates was measured with the Pierce BCA Protein Assay Kit (Thermofisher Scientific, Rockford, IL) per the manufacturer's protocol. The same concentration of protein was utilized for each sample. Precleared cell lysates were incubated with anti-SOD3 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hour at 4 °C, followed by precipitation with 20 μl of Protein A/G PLUS-Agarose (Santa Cruz Biotechnology) and incubated at 4 °C overnight. Adherent proteins were eluted with 1×protein loading buffer for 5 minutes at 80 °C and analyzed by a Western blot

detection kit (Visualizer, Millipore, Temecula, CA). SOD3 bands were scanned and analyzed by NIH Image J 3.0 (.U. S. National Institutes of Health, Bethesda,Maryland).

Measurement of HIF-1α levels in EOC cells

Protein concentration of cell lysates was measured as described above. Cell lysates were prepared from the various treatments of the EOC cell lines, SKOV-3 and MDAH-2774. HIF-1α was measured with an enzyme-linked immunosorbent assay kit (HIF-1α ELISA, R&D Systems, Minneapolis, MN), per the manufacturer's protocol.

Immunohistochemical staining of EOC tissue sections

Twenty benign ovarian tissues specimens, obtained from patients who underwent total abdominal hysterectomy-bilateral salpingoooophorectomy for leiomyomas, and 20 invasive EOC cases (two sections per case) were retrieved from archival materials from the Detroit Medical Center/ Karmanos Cancer Center pathology department (Institutional review board number 072206MP2E). Histological diagnoses were as follows: 10 high grade and five low grade serous carcinomas, four grade 1 endometrioid and one grade 1 mucinous cacinomas. The mean age of the 20 patients was 56 (range 35–70). Sections were deparaffinized, hydrated with PBS (pH 7.4), and pretreated with H_2O_2 (3%) for 10 min to remove endogenous peroxidases and incubated in goat serum for 10 min. A primary antibody for NADPH oxidase (HPA015475, Sigma Aldrich) dilution (1:20) was applied to each section, followed by washing and incubation with the biotinylated secondary antibody for 10 min at room temperature. Detection was performed with AEC and counter-staining with Mayer's hematoxylin followed by mounting.

Ovaries had only surface epithelial inclusion cysts and epithelium was evaluated for expression of NADPH oxidase and was assessed based on the presence of cytoplasmic staining. Scoring was assigned based on the percentage of positive epithelial cells: a zero score assigned for cases with no cytoplasmic staining in any cells; score 1 with b5% of cell staining positive; score 2 with 6-30% and score 3 with N30% of cells staining positive. A gynecologic pathologist reviewed all slides, benign (2–5 slides) and malignant (5–15 slides). The most positive area in every tumor or benign cases was evaluated and only the percentage was evaluated because most of the cases had the same intensity. For statistical analysis, cases with score 0 or 1 were considered as being negative and cases with score 2 or 3 as positive.

Statistical Analysis

Data were analyzed using SPSS 19.0 for windows (SPSS for Windows, Chicago, IL). Data were analyzed using oneway ANOVA (analysis of variance) with Student Neuman-Kuels post-hoc comparisons. Significance values of $p<0.05$ were considered statistically significant for all analyses.

Jiang et al.   Page 6

Results

DPI treated EOC cells exhibited increased caspase-3 activity and apoptosis

Caspase-3 activity significantly increased, in a time dependent fashion, in SKOV-3 cells, from 7.62 to 20.9, 20.3, 22.0, 24.5, 39.4, and 54.3 μM and in MDAH-2774 cells from 6.61 to 19.0, 20.4, 23.1, 25.6, 39.3, and 53.1 μM at the 0.5, 1, 3, 6, 12, and 24 hour time points, respectively (pb0.05 as compared to control, Fig. 1A).

These results were confirmed by TUNEL staining, an indicator of the degree of DNA fragmentation, which is representative of apoptosis. Nuclei were stained with DAPI (blue) and apoptotic cells were visualized (60x) with fluorescein-12-dUTP (green). There was a significant increase in TUNEL staining (green) as compared to controls, in both EOC cell lines (Fig. 1B).

DPI treated EOC cells had reduced levels of NADPH oxidase mRNA

Real-time RT-PCR was utilized to determine the mRNA level of the NADPH oxidase p22-phox subunit, a representative O_2 sensing subunit of NADPH oxidase, in EOC cells treated with and without DPI for 0.5 hours. NADPH oxidase was significantly decreased by 51.6% in SKOV-3 cells, and by 40.1% in MDAH-2774 cells ($p < 0.05$ as compared to control, Fig. 2).

EOC tissues expressed higher levels of NADPH oxidase

NADPH oxidase expression was upregulated in 65% of EOC tissue sections, tested by immunohistochemistry as compared to 20% detectable expression for NADPH oxidase in normal ovarian epithelial tissue (surface epithelial inclusion cysts) (Fig. 3).

DPI treated EOC cells had reduced levels of HIF-1α

DPI treatment significantly reduced HIF-1α mRNA levels, in SKOV-3 cells, from 428.2 to 368.2, 318.6, 268.8, 261.3, and 206.8 pg/μg RNA at the 0.5, 3, 6, 12, and 24 hour time points, and in MDAH-2774 cells from 421.3 to 356.9, 356.1, 327.4, 260.3, 240.0 and 223.1 pg/μg RNA, at the 0.5, 1, 3, 6, 12, and 24 hour time points, respectively ($p < 0.05$ as compared to control, Fig. 4A). There was no statistical change in HIF-1α mRNA levels at the 1 hour time point in SKOV-3 cells.

HIF-1α protein levels, in SKOV-3 cells, were significantly reduced from 1699 to 828.0, 377.2, 311.0, 291.1, 224.6, and 44.27 pg/ml total protein and in MDAH-2774 cells from 1872 to 658.1, 332.4, 257.5, 111.8, 106.1, and 22.96 pg/ml total protein at the 0.5, 1, 3, 6, 12, and 24 hour time points, respectively ($p < 0.05$ as compared to control, Fig. 4B).

DPI treated EOC cells had reduced levels of SOD3

DPI treatment significantly reduced SOD3 mRNA levels, in a time dependent fashion, from 31.1 to 19.5, 24.6, 15.7, 9.23, 4.63, and 3.41 fg/μg RNA at the 0.5, 1, 3, 6, 12, and 24 hour time points, respectively, in SOKV-3 cells ($p < 0.05$ as compared to control, Fig. 5A). Similarly, SOD3 mRNA levels were significantly reduced from 26.1 to 19.69, 16.0, 11.7, 8.63, and 6.68 fg/μg RNA at the 0.5, 3, 6, 12, and 24 hour time points, respectively, in

MDAH-2774 cells ($p<0.05$ as compared to control, Fig. 5A). There was no significant change in SOD3 mRNA levels at the 1 hour time point in MDAH-2774 cells.

SOD3 protein levels, in SOKV-3 cells, were reduced from 251.6 to 223.2, 215.7, 181.6, and 149.0 at the 3, 6, 12, and 24 hour time points, respectively ($p<0.05$ as compared to control, Figs. 5B and C) and in MDAH-2774 cells from 254.4 to 237.9, 230.4, 208.5, 188.4, and 170.1 at the 1, 3, 6, 12, and 24 hour time points, respectively ($p<0.05$ as compared to control, Figs. 5B and C). There was no significant change in SOD3 protein levels at the 0.5 hour time point in both SKOV-3 and MDAH-2774 cells and at the 1 hour time point in SKOV-3 cells. Results were expressed as relative levels as compared to their control.

Discussion

In this study we sought to determine the effects of inhibiting the generation of ROS by DPI, a well-characterized, potent inhibitor of flavoenzymes including NADPH oxidase, on apoptosis of EOC cells, and whether these effects are associated with SOD3 and HIF-1α expression [30,31]. Diphenyleneiodonium has been used to inhibit ROS production mediated by NADPH oxidase in various cell types [21,31,32]. Our immunohistochemical results showed that NADPH oxidase is over-expressed in EOC tissues as compared to normal ovarian tissues (Fig. 3). Consistent with this observation, we demonstrated EOC cells to have elevated NADPH oxidase, which was reduced by DPI (Fig. 2). These findings are supported by the fact that increased NADPH oxidase levels promote the tumorigenic potential of NIH3T3 mouse fibroblasts as well as the DU-145 prostate epithelial cells [30].

Inhibition of NADPH oxidase has been reported to significantly limit the conversion of molecular O_2 to $O_2^{\cdot -}$, H_2O_2 and other ROS [24,25]. Growing evidence suggests that cancer cells exhibit increased intrinsic ROS stress, due in part to oncogenic stimulation, increased metabolic activity, and mitochondrial malfunction [2,3]. Further support for this increase in ROS is demonstrated by a cross-talk between mitochondria and the $O_2^{\cdot -}$ generating NADPH oxidase in ovarian tumors [33]. The mitochondria controls NADPH oxidase redox signaling, therefore loss of this control contributes to tumorigenesis [33]. In agreement with a previous study, we have shown that inhibition of NADPH oxidase-dependent ROS generation with DPI induced apoptosis in EOC cells, suggesting that the ROS produced by NADPH oxidase, at least in part, exert an anti-apoptotic function [34]. This anti-apoptotic mechanism involves induced inhibition of phosphorylation of AKT and subsequent suppression of AKT-mediated phosphorylation of ASK1 on Ser-83 [34–36]. Furthermore, the anticancer drug paclitaxel-induced apoptosis of ovarian cancer cells is mediated by negative regulation of AKT–ASK1 phosphorylation signaling whereas AKT activation by H_2O_2 confers protection against apoptosis [34–36].

In addition, we have shown that DPI treatment significantly reduced SOD3 and HIF-1α mRNA levels as early as 30 minutes after treatment, with significant further reduction over the following 24 hours in EOC cells (Figs. 4 and 5). A parallel reduction in protein levels, although not with equivalent magnitude, was also observed for both SOD3 and HIF-1α as determined by IP/Western blot and ELISA (Figs. 4 and 5). This may be a consequence of post-translation modifications, which may result in increased stability and/or lower

Jiang et al.   Page 8

degradation of the proteins. These findings demonstrated that inhibition of NADPH oxidase attenuates the expression of both SOD3 and HIF-1α, at the mRNA and protein levels. Moreover, our results showed that the inhibition of NADPH oxidase significantly induced apoptosis of EOC cells, as assessed by both caspase-3 activity and TUNEL assays (Fig. 1). Therefore, there appears to be a strong association between the inhibition of NADPH oxidase and the subsequent reduction in SOD3 and HIF-1α levels and increase in apoptosis of EOC cells.

The correlation between HIF-1α and cellular apoptosis has previously been demonstrated in lung and hepatoma cancer cells [25,37]. Apoptosis can regulate HIF-1α through the modulation of the ratio of pro-apoptotic Bcl-2 and anti-apoptotic Bcl-2 family proteins [38]. Anti-apoptotic Bcl-2 and Bcl-xL levels were increased and proapoptotic BAX and BAK levels were decreased with the over-expression of HIF-1α [38]. Also, it has been reported that inhibition of HIF-1α by rapamycin resulted in an increase in apoptosis of cancer cells, and decrease in the expression of apoptosis inhibitor Bcl-2 in ovarian cancer xenografts and that rapamycin enhanced cell death through the inhibition of cell survival signals in a number of cell lines [39].

Most of the generated $O_2^{\cdot-}$ undergoes a nonenzymatic or SOD-catalyzed reaction, generating H_2O_2 as an end product [40–42]. Hydrogen peroxide is freely diffusible through biological membranes, and its overproduction is extremely destructive to cells and tissues, yet it is physiologically important among ROS given its relative long half-life in the intracellular space, and that it is the precursor of the more toxic hydroxyl radicals [41–43]. It has been reported that increased SOD3 expression in ovarian cancer is a cellular response to intrinsic ROS stress [44]. However, the role of SOD3 in tumorigenesis is somewhat controversial. It has been recently demonstrated, in mice, that subcutaneous inoculation of the SOD3 gene significantly suppressed lung cancer metastasis and that over-expression of SOD3 resulted in *in vivo* inhibition of growth of B16-F1 melanoma tumors [45,46]. In contrast, inhibition of SOD has been shown to selectively induce apoptosis of leukemia and ovarian cancer cells, confirming our findings from the present study [5].

High expression levels of SOD3 was reported to significantly induce the expression of HIF-1α in tumors, under hypoxic conditions, and thus demonstrates a relationship between SOD3 and HIF-1α pathways [39]. The mechanism by which SOD3 upregulates HIF-1α is not well understood, but there is substantial evidence to suggest that this mechanism is modulated, in part, by the steady-state level of $O_2^{\cdot-}$ and the stabilization of HIF-1α [47]. Therefore, reduction of $O_2^{\cdot-}$ levels via inhibition of NADPH oxidase may result in lowering SOD3 levels, leading to the destabilization of HIF-1α, subsequently increasing apoptosis.

Collectively, based on previously discussed published reports and the results from this study, we conclude that lowering oxidative stress, possibly through the inhibition of NADPH oxidase-generated $O_2^{\cdot-}$, induces apoptosis in ovarian cancer cells and may serve as a potential target for cancer therapy.

Jiang et al. Page 9

References

1. Jemal A, Siegel R, Ward E, Murray T, Xu J, Smigal C, et al. Cancer statistics, 2006. CA Cancer J Clin. 2006; 56(2):106–30. [PubMed: 16514137]

2. Trachootham D, Alexandre J, Huang P. Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach? Nat Rev Drug Discov. 2009; 8(7):579–91. [PubMed: 19478820]

3. Pelicano H, Carney D, Huang P. ROS stress in cancer cells and therapeutic implications. Drug Resist Updat. 2004; 7(2):97–110. [PubMed: 15158766]

4. Starkov AA, Fiskum G, Chinopoulos C, Lorenzo BJ, Browne SE, Patel MS, et al. Mitochondrial alpha-ketoglutarate dehydrogenase complex generates reactive oxygen species. J Neurosci. 2004; 24(36):7779–88. [PubMed: 15356189]

5. Hileman EO, Liu J, Albitar M, Keating MJ, Huang P. Intrinsic oxidative stress in cancer cells: a biochemical basis for therapeutic selectivity. Cancer Chemother Pharmacol. 2004; 53(3):209–19. [PubMed: 14610616]

6. Cheng G, Cao Z, Xu X, van Meir EG, Lambeth JD. Homologs of gp91phox: cloning and tissue expression of Nox3, Nox4, and Nox5. Gene. 2001; 269(1–2):131–40. [PubMed: 11376945]

7. Ushio-Fukai M, Alexander RW. Reactive oxygen species as mediators of angiogenesis signaling: role of NAD(P)H oxidase. Mol Cell Biochem. 2004; 264(1–2):85–97. [PubMed: 15544038]

8. Bedard K, Krause KH. The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. Physiol Rev. 2007; 87(1):245–313. [PubMed: 17237347]

9. Dong J, Sulik KK, Chen SY. The role of NOX enzymes in ethanol-induced oxidative stress and apoptosis in mouse embryos. Toxicol Lett. 2010; 193(1):94–100. (PMCID: 2822117). [PubMed: 20026259]

10. Meier B, Cross AR, Hancock JT, Kaup FJ, Jones OT. Identification of a superoxide-generating NADPH oxidase system in human fibroblasts. Biochem J. 1991; 275(Pt 1):241–5. (PMCID: 1150038). [PubMed: 1850240]

11. Szatrowski TP, Nathan CF. Production of large amounts of hydrogen peroxide by human tumor cells. Cancer Res. 1991; 51(3):794–8. [PubMed: 1846317]

12. Griendling KK, Minieri CA, Ollerenshaw JD, Alexander RW. Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells. Circ Res. 1994; 74(6):1141–8. [PubMed: 8187280]

13. Lambeth JD. NOX enzymes and the biology of reactive oxygen. Nat Rev Immunol. 2004; 4(3):181–9. [PubMed: 15039755]

14. Fletcher NM, Jiang ZL, Diamond MP, Abu-Soud HM, Saed GM. Hypoxia-generated superoxide induces the development of the adhesion phenotype. Free Radic Biol Med. 2008; 45(4):530–6. (PMCID: 2574925). [PubMed: 18538674]

15. Fridovich I. The biology of oxygen radicals. Science. 1978; 201(4359):875–80. [PubMed: 210504]

16. Carlsson LM, Jonsson J, Edlund T, Marklund SL. Mice lacking extracellular superoxide dismutase are more sensitive to hyperoxia. Proc Natl Acad Sci U S A. 1995; 92(14):6264–8. [PubMed: 7603981]

17. Chaiswing L, Zhong W, Cullen JJ, Oberley LW, Oberley TD. Extracellular redox state regulates features associated with prostate cancer cell invasion. Cancer Res. 2008; 68(14):5820–6. [PubMed: 18632636]

18. Semenza GL. Hypoxia-inducible factor 1: control of oxygen homeostasis in health and disease. Pediatr Res. 2001; 49(5):614–7. [PubMed: 11328942]

19. Takeya R, Sumimoto H. Molecular mechanism for activation of superoxide-producing NADPH oxidases. Mol Cells. 2003; 16(3):271–7. [PubMed: 14744014]

20. Chandel NS, Maltepe E, Goldwasser E, Mathieu CE, Simon MC, Schumacker PT. Mitochondrial reactive oxygen species trigger hypoxia-induced transcription. Proc Natl Acad Sci U S A. 1998; 95(20):11715–20. [PubMed: 9751731]

21. Suliman HB, Ali M, Piantadosi CA. Superoxide dismutase-3 promotes full expression of the EPO response to hypoxia. Blood. 2004; 104(1):43–50. [PubMed: 15016652]

Jiang et al.   Page 10

22. Cross AR, Jones OT. The effect of the inhibitor diphenylene iodonium on the superoxide-generating system of neutrophils. Specific labelling of a component polypeptide of the oxidase. Biochem J. 1986; 237(1):111–6. [PubMed: 3800872]

23. Saed GM, Zhao M, Diamond MP, Abu-Soud HM. Regulation of inducible nitric oxide synthase in post-operative adhesions. Hum Reprod. 2006; 21(6):1605–11. [PubMed: 16484312]

24. Boveris A, Chance B. The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen. Biochem J. 1973; 134(3):707–16. [PubMed: 4749271]

25. Volm M, Koomagi R. Hypoxia-inducible factor (HIF-1) and its relationship to apoptosis and proliferation in lung cancer. Anticancer Res. 2000; 20(3A):1527–33. [PubMed: 10928066]

26. Brault S, Gobeil F Jr, Fortier A, Honore JC, Joyal JS, Sapieha PS, et al. Lysophosphatidic acid induces endothelial cell death by modulating the redox environment. Am J Physiol Regul Integr Comp Physiol. 2007; 292(3):R1174–83. [PubMed: 17122328]

27. O'Donnell BV, Tew DG, Jones OT, England PJ. Studies on the inhibitory mechanism of iodonium compounds with special reference to neutrophil NADPH oxidase. Biochem J. 1993; 290(Pt 1):41–9. (PMCID: 1132380). [PubMed: 8439298]

28. Park SE, Song JD, Kim KM, Park YM, Kim ND, Yoo YH, et al. Diphenyleneiodonium induces ROS-independent p53 expression and apoptosis in human RPE cells. FEBS Lett. 2007; 581(2): 180–6. [PubMed: 17184774]

29. Suh YA, Arnold RS, Lassegue B, Shi J, Xu X, Sorescu D, et al. Cell transformation by the superoxide-generating oxidase Mox1. Nature. 1999; 401(6748):79–82. [PubMed: 10485709]

30. Arbiser JL, Petros J, Klafter R, Govindajaran B, McLaughlin ER, Brown LF, et al. Reactive oxygen generated by Nox1 triggers the angiogenic switch. Proc Natl Acad Sci U S A. 2002; 99(2): 715–20. [PubMed: 11805326]

31. Tanaka M, Kogawa K, Nakamura K, Nishihori Y, Kuribayashi K, Hagiwara S, et al. Anti-metastatic gene therapy utilizing subcutaneous inoculation of EC-SOD gene transduced autologous fibroblast suppressed lung metastasis of Meth-A cells and 3LL cells in mice. Gene Ther. 2001; 8(2):149–56. [PubMed: 11313784]

32. Wheeler MD, Smutney OM, Samulski RJ. Secretion of extracellular superoxide dismutase from muscle transduced with recombinant adenovirus inhibits the growth of B16 melanomas in mice. Mol Cancer Res. 2003; 1(12):871–81. [PubMed: 14573788]

33. Desouki MM, Kulawiec M, Bansal S, Das GM, Singh KK. Cross talk between mitochondria and superoxide generating NADPH oxidase in breast and ovarian tumors. Cancer Biol Ther. 2005; 4(12):1367–73. [PubMed: 16294028]

34. Mochizuki T, Furuta S, Mitsushita J, Shang WH, Ito M, Yokoo Y, et al. Inhibition of NADPH oxidase 4 activates apoptosis via the AKT/apoptosis signal-regulating kinase 1 pathway in pancreatic cancer PANC-1 cells. Oncogene. 2006; 25(26):3699–707. [PubMed: 16532036]

35. Mabuchi S, Ohmichi M, Kimura A, Nishio Y, Arimoto-Ishida E, Yada-Hashimoto N, et al. Estrogen inhibits paclitaxel-induced apoptosis via the phosphorylation of apoptosis signal-regulating kinase 1 in human ovarian cancer cell lines. Endocrinology. 2004; 145(1):49–58. [PubMed: 14500571]

36. Wang X, McCullough KD, Franke TF, Holbrook NJ. Epidermal growth factor receptor-dependent Akt activation by oxidative stress enhances cell survival. J Biol Chem. 2000; 275(19):14624–31. [PubMed: 10799549]

37. Piret JP, Mottet D, Raes M, Michiels C. CoCl2, a chemical inducer of hypoxiainducible factor-1, and hypoxia reduce apoptotic cell death in hepatoma cell line HepG2. Ann N Y Acad Sci. 2002; 973:443–7. [PubMed: 12485908]

38. Li Y, Trush MA. Diphenyleneiodonium, an NAD(P)H oxidase inhibitor, also potently inhibits mitochondrial reactive oxygen species production. Biochem Biophys Res Commun. 1998; 253(2): 295–9. [PubMed: 9878531]

39. Shi Y, Frankel A, Radvanyi LG, Penn LZ, Miller RG, Mills GB. Rapamycin enhances apoptosis andincreases sensitivity to cisplatin in vitro. Cancer Res. 1995; 55(9):1982–8. [PubMed: 7728769]

40. Scaife RM. Selective and irreversible cell cycle inhibition by diphenyleneiodonium. Mol Cancer Ther. 2005; 4(6):876–84. [PubMed: 15956245]

Jiang et al.   Page 11

41. Goud AP, Goud PT, Diamond MP, Gonik B, Abu-Soud HM. Reactive oxygen species and oocyte aging: role of superoxide, hydrogen peroxide, and hypochlorous acid. Free Radic Biol Med. 2008; 44(7):1295–304. [PubMed: 18177745]

42. McCord JM, Fridovich I. Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). J Biol Chem. 1969; 244(22):6049–55. [PubMed: 5389100]

43. Schallreuter KU, Moore J, Wood JM, Beazley WD, Gaze DC, Tobin DJ, et al. In vivo and in vitro evidence for hydrogen peroxide (H2O2) accumulation in the epidermis of patients with vitiligo and its successful removal by a UVB-activated pseudocatalase. J Investig Dermatol Symp Proc. 1999; 4(1):91–6.

44. Hu Y, Rosen DG, Zhou Y, Feng L, Yang G, Liu J, et al. Mitochondrial manganesesuperoxide dismutase expression in ovarian cancer: role in cell proliferation and response to oxidative stress. J Biol Chem. 2005; 280(47):39485–92. [PubMed: 16179351]

45. Calastretti A, Bevilacqua A, Ceriani C, Vigano S, Zancai P, Capaccioli S, et al. Damaged microtubules can inactivate BCL-2 by means of the mTOR kinase. Oncogene. 2001; 20(43):6172–80. [PubMed: 11593425]

46. Jiang H, Feng Y. Hypoxia-inducible factor 1alpha (HIF-1alpha) correlated with tumor growth and apoptosis in ovarian cancer. Int J Gynecol Cancer. 2006; 16(Suppl 1):405–12. [PubMed: 16515634]

47. Kaewpila S, Venkataraman S, Buettner GR, Oberley LW. Manganese superoxide dismutase modulates hypoxia-inducible factor-1 alpha induction via superoxide. Cancer Res. 2008; 68(8):2781–8. [PubMed: 18413745]

Jiang et al. Page 12
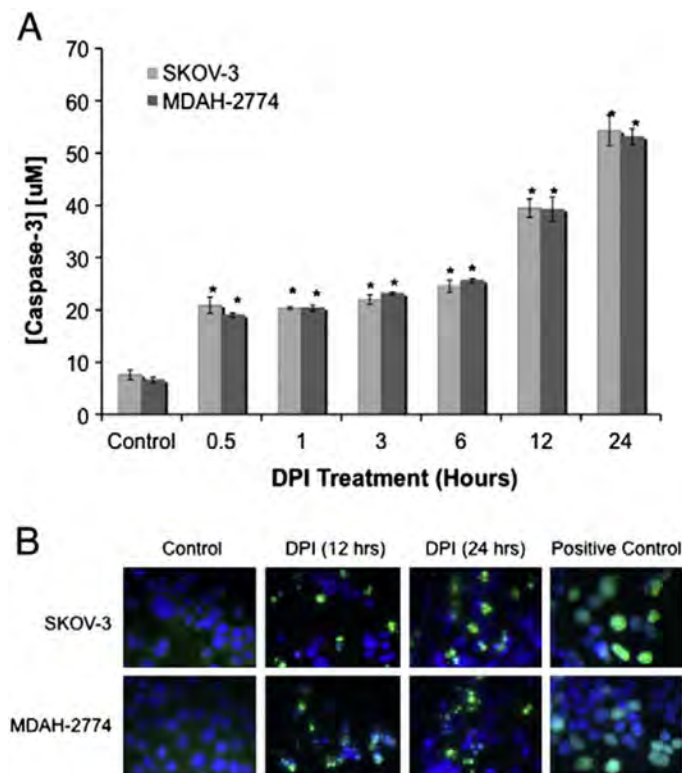
Fig. 1.
(A) Caspase-3 activity and (B) apoptosis in EOC cells. (A) Caspase-3 activity was measured in cell lysates from SKOV-3 and MDAH-2774 before and after DPI treatment (10 μM) at various time points. (B) The amount of DNA fragmentation (apoptosis) was assessed by TUNEL assay in MDAH-2774 and SKOV-3, before and after DPI treatment (10 μM, 12 and 24 hrs) as compared to control cells. Nuclei were stained with DAPI (blue) and apoptotic cells were visualized (60x) with fluorescein-12-dUTP (green). Results are representative of the mean of three independent experiments. (* *All p<0.05 as compared to their control*).
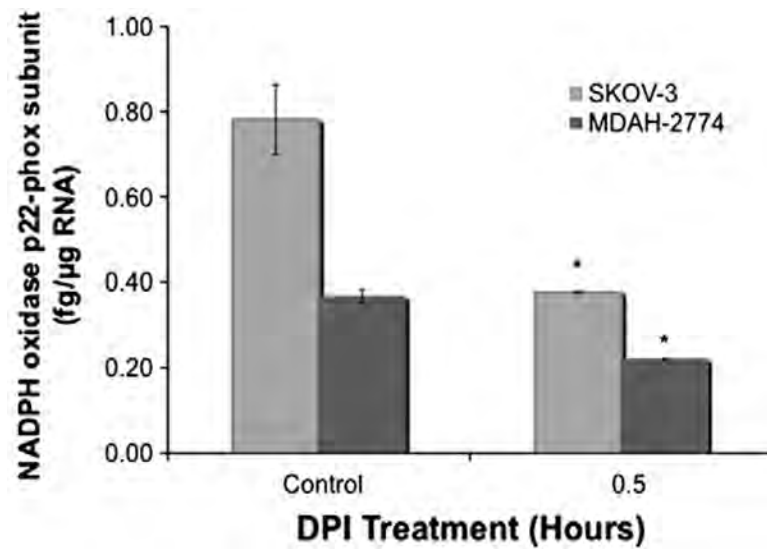
Jiang et al.

Page 13

Fig. 2.
Real-time RT-PCR for NADPH oxidase in EOC cells. Expression of the NADPH oxidase subunit p22-*phox* mRNA levels in SKOV-3 and MDAH-2774 before and after DPI treatment (10 μM, 0.5 hrs) was measured using real-time RT-PCR. Results are representative of the mean of three independent experiments. (* *All p<0.05 as compared to their control*).

Jiang et al.

Page 14

Fig. 3.
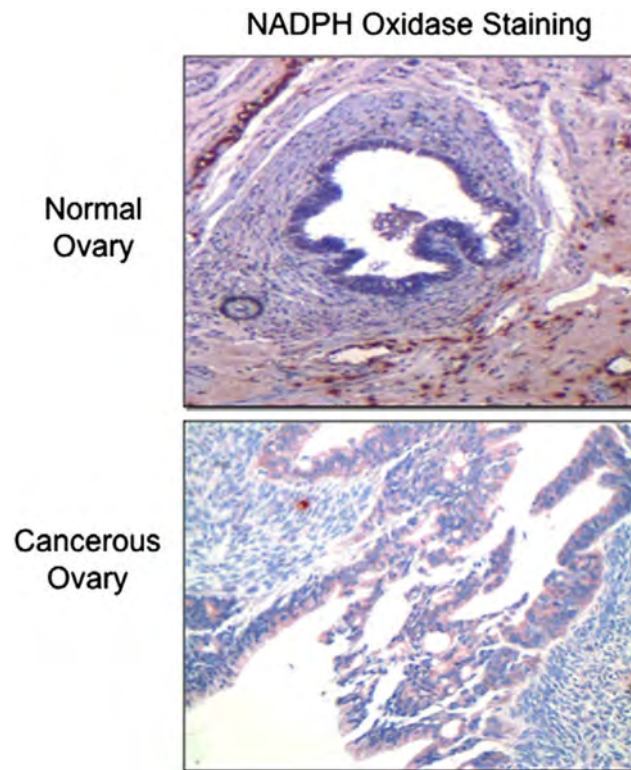Immunohistochemical staining of EOC tissue sections. Normal and EOC tissue section were stained with a primary antibody for NADPH oxidase, followed by biotinylated secondary antibody. Detection was performed with AEC and counter-staining was done with Mayer's hematoxylin followed by mounting and imaging (20x). The expression of NADPH oxidase was assessed based on the presence of cytoplasmic staining.

Jiang et al. Page 15
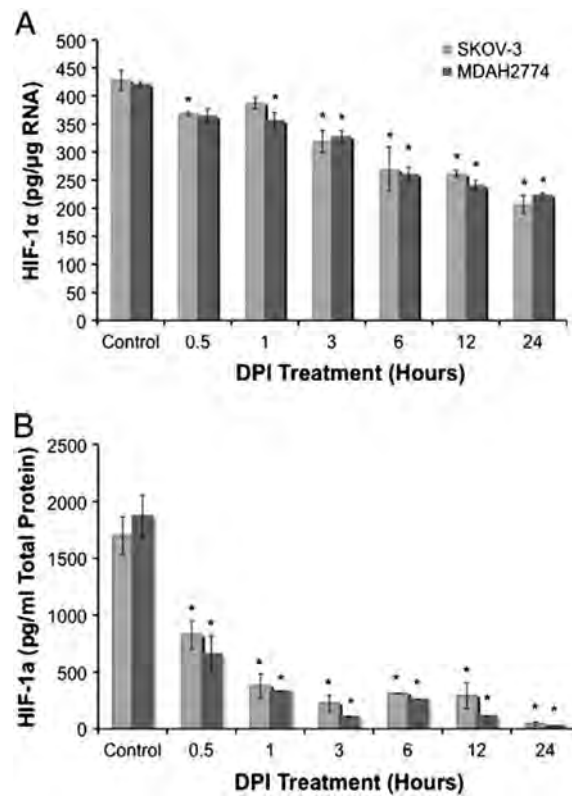
Fig. 4.
HIF-1α levels in EOC cells. (A) HIF-1α mRNA levels in SKOV-3 and MDAH-2774 before and after DPI treatment (10 μM), at various time points, were measured using real-time RT-PCR. (B) ELISA was performed at different time points for cell lysates from SKOV-3 and MDAH-2774 before and after DPI treatment (10 μM) at various time points. Results are representative of the mean of three independent experiments. Results are representative of the mean of three independent experiments. (* *All p<0.05 as compared to their control*).

Jiang et al.

Page 16
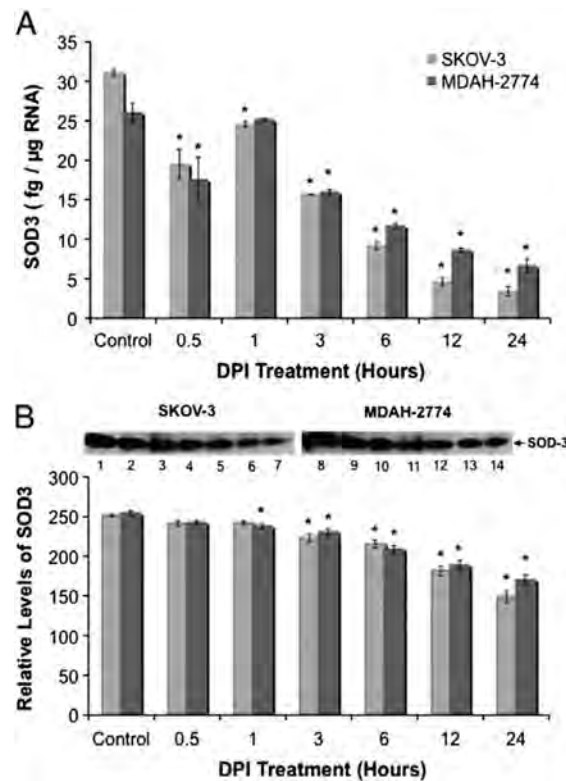
Fig. 5.
SOD3 levels in EOC cells. (A) SOD3 mRNA levels in SKOV-3 and MDAH-2774 before and after DPI treatment (10 μM), at various time points, were measured using real-time RT-PCR. (B) IP/Western blot was utilized to detect SOD3 protein levels in EOC cells. Cell lysates from SKOV-3 and MDAH-2774 before and after DPI treatment (10 μM), at various time points, were precipitated with anti-SOD3 antibody and fractionated with SDS-PAGE. Membrane was probed with anti-SOD3 antibody. Immunoprecipitation of SOD3 exposed to SOD3 antibody are as follows: Lanes 1 & 8; control, Lanes 2 & 9; 0.5 hr, Lanes 3 & 10; 1 hr, Lanes 4 & 11; 3 hr, Lanes 5 & 12; 6 hr, Lanes 6 & 13; 12 hr, and Lanes 7 & 14; 24 hr. (C): IP/Western blot results were scanned and analyzed by NIH image J 3.0. Results are representative of the mean of three independent experiments. (* *All p<0.05 as compared to their control*).

Jiang et al. Page 17

Table 1

Sequence of Human Oligonucleotide Primers.

Locus	Sense (5′-3′)	Antisense (5′-3′)	Product Length (bp)
HIF-1α	AGCCGAGGAAGAACTATGAAC	ACTGAGGTTGGTTACTGTTGG	100
p22-*phox*	GTACTTTGGTGCTTACTC	GGAGCCCTTTTTCCTCTT	82
SOD3	GCCTCCATTTGTACCGAAAC	AGGGTCTGGGTGGAAAGG	78

Exhibit 84

Original Article

The Role of Oxidative Stress in the Development of Cisplatin Resistance in Epithelial Ovarian Cancer

Jimmy Belotte, MD[1], Nicole M. Fletcher, PhD[1], Awoniyi O. Awonuga, MD[1], Mitchell Alexis, BS[2], Husam M. Abu-Soud, PhD[1], Mohammed G. Saed[1], Michael P. Diamond, MD[3], and Ghassan M. Saed, PhD[1]

Abstract

Objective: To investigate the role of oxidative stress in the development of cisplatin resistance in epithelial ovarian cancer (EOC). **Methods:** Two parent EOC cell lines (MDAH-2774 and SKOV-3) and their chemoresistant counterparts (cisplatin, 50 µmol/L) were used. Total RNA was extracted and subjected to real-time reverse transcriptase polymerase chain reaction to evaluate the expression of glutathione reductase (GSR) and inducible nitric oxide synthase (iNOS), as well as nitrate/nitrite levels. Analysis of variance was used for main effects and Tukey for post hoc analysis at $P < .05$ for statistical significance. **Results:** Both cisplatin resistant cell lines displayed a significant decrease in GSR messenger RNA (mRNA) levels and activity ($P < .01$). As compared to sensitive controls, nitrate/nitrite levels were significantly higher in SKOV-3 cisplatin resistant cells while iNOS mRNA levels were significantly higher in MDAH-2774 cisplatin resistant cells ($P < .05$). **Conclusion:** Our data suggest that the development of cisplatin resistance *tilts the balance toward* a *pro-oxidant state* in EOC.

Keywords

epithelial ovarian cancer, cisplatin resistance, reactive oxygen species

Introduction

Ovarian cancer is the principal cause of death of all gynecologic cancers, with an estimated 22 240 new cases and 14 030 deaths expected in 2013 in the United States alone.[1] Over the past several decades, the chemotherapeutic management of epithelial ovarian cancer (EOC) has significantly evolved from single alkylating agent to platinum-taxane combinations and bevacizumab, more recently. Unfortunately, between 60% and 80% of those who initially responded will relapse with a platinum-free interval at less than 6 months and therefore are considered platinum resistant.[2]

Reactive oxygen species (ROS) are known to be involved in many physiological processes such as redox signaling and immunity, as well as pathological processes such as cancer, through the promotion of genetic instability, abnormal cell proliferation, and angiogenesis.[3-5] The nicotinamide adenine dinucleotide phosphate (NADPH) oxidase family, a membrane-bound enzyme complex, is a key player in the generation of ROS as well as inducible nitric oxide synthase (iNOS).[5-8] Physiologic pathways involving crucial enzymes such as glutathione reductase (GSR), glutathione peroxidase, catalase, and superoxide dismutase consume free radicals and other oxidants, thus assisting in the maintenance of oxidative balance. When these latter processes are overwhelmed, a state of oxidative stress ensues. Ultimately, this pervasive environment can damage all components of the cell, including proteins, lipids, and DNA.[9,10] Paradoxically, evidence suggests that cancer cells can take advantage of these processes to evade the immune system, avoid apoptosis, and become resistant to chemotherapy.[11]

The rationale of this research proposal is that cancer cells function under significant intrinsic oxidative stress using glycolytic metabolism even in the presence of high oxygen availability (Warburg effect).[12,13] Previous studies have demonstrated that alterations in redox signaling pathways are involved in the pathophysiology of EOC.[5,6,14,15] Reportedly, increasing iNOS activity can lead to S-nitrosylation of caspase-3 and decreased

[1] Department of Obstetrics and Gynecology, C.S. Mott Center for Human Growth and Development, Wayne State University School of Medicine, Detroit, MI, USA
[2] Office of Graduate Scholars, Wayne State University School of Medicine, Detroit, MI, USA
[3] Department of Obstetrics and Gynecology, Georgia Regents University, Agusta, GA, USA

Corresponding Author:
Ghassan M. Saed, Department of Obstetrics and Gynecology, C. S. Mott Center for Human Growth and Development, Wayne State University School of Medicine, 275 E. Hancock, Detroit, MI 48201, USA.
Email: gsaed@med.wayne.edu

Table 1. Human Oligonucleotide Primers.

Locus	Sense (5′ 3′)	Antisense (3′ 5′)	Size, bp
GSR	TCACCAAGTCCCATATAGAAATC	TGTGGCGATCAGGATGTG	116
iNOS	GGCACAGAACTTAAGGATGG	TTGTTAGGAGGTCAAGTAAAGG	145

Abbreviations: bp, base pair; GSR, glutathione reductase; iNOS, inducible nitric oxide synthase.

apoptosis in EOC cells.[5] Moreover, many have evidence outlining the role of glutathione and GS-XlMRPP2 efflux pumps as one of the mechanisms of cisplatin resistance.[16-18] Discovering the exact mechanisms involving the main players of ROS metabolism and their relationship to resistance to chemotherapy will provide new insights into tumor biology and allow the identification of new therapeutic targets that can be swiftly translated to clinical interventions. Our objective in this study was to investigate the role of oxidative stress in the development of cisplatin resistance in EOC cells.

Materials and Methods

Cell Culture

Two parent human EOC cell lines (SKOV-3 and MDAH-2774 from American Type Culture Collection, Manassas, Virginia) were utilized in developing cells resistant to cisplatin. Cells were exposed to a stepwise increase in cisplatin (Sigma Aldrich, St Louis, Missouri) over the course of a year, with final concentrations of 50 μmol/L. The Trypan Blue Dye Exclusion Method was used to confirm resistance as follows: once the desired level of resistance of 50 μmol/L was achieved, a 2-week incubation period without cisplatin and subsequent reintroduction of cisplatin was performed. Cells were cultured in 60-mm^2 dishes with McCoy's 5A medium (Invitrogen, Grand Island, New York) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, Georgia) and 1% penicillin and streptomycin (Invitrogen).

For measurement of GSR messenger RNA (mRNA) and activity and nitrate/nitrite levels, cells (2.5×10^6) were seeded in 150-mm dishes (Corning, Corning, New York) and allowed to rest for 24 hours followed by media replacement and cell collection 24 hours later.

For measurement of iNOS mRNA levels, cells (1×10^6) were seeded in 150 mm^3 dishes (Corning) and cultured over 72 hours.

Real-Time Reverse Transcription Polymerase Chain Reaction

RNA isolation. Total RNA was extracted from EOC cells using the RNeasy Mini Kit (Qiagen, Valencia, California) according to the protocol provided by the manufacturer.

Reverse transcription. A 40-μL-complementary DNA (cDNA)-reaction volume utilizing 2 μg RNA was prepared using the QuantiTect Reverse Transcription Kit (Qiagen), as described by the manufacturer's protocol.

Real-time RT-PCR primer design and controls. Optimal oligonucleotide primer pairs for real-time reverse transcription polymerase chain reaction (RT-PCR) amplification of reverse-transcribed cDNA were selected with the aid of the software program, Beacon Designer (Premier Biosoft Int, Palo Alto, California). Human oligonucleotide primers, which amplify variable portions of the protein coding regions, were used. Sequences of the oligonucleotides used for amplification of iNOS and GSR are described in Table 1.

Quantitative real-time RT-PCR was performed using a Quanti-Tect SYBR Green RT-PCR kit (Qiagen) and Cepheid 1.2f Detection System (Cepheid, Sunnyvale, California). The RT-PCR was performed in a 25 μL total reaction volume including 12.5 μL of 2× QuantiTect SYBR Green RT-PCR master mix, 5 μL of 10× diluted cDNA template for GSR and 2 μL of undiluted cDNA template for iNOS, and 0.2 μmol/L each of target specific primers designed to amplify each gene. Standards with known concentrations and lengths (base pairs [bp]) were designed specifically for iNOS [103 bp] and GSR [103 bp] using the Beacon Designer software (Premier Biosoft), allowing for construction of a standard curve using a 10-fold dilution series. A specific standard for each gene allows for absolute quantification of the gene in a number of copies, which can then be expressed per μg of RNA. The RT-PCR conditions were programmed as follows: an initial cycle was performed at 95°C for 1000 seconds for iNOS and 900 seconds for GSR. This was followed by 35 cycles of denaturation at 95°C for 15 seconds, annealing for 30 seconds at 58°C for iNOS, and 59°C for GSR. This was followed by a final cycle at 72°C for 30 seconds to allow completion of product synthesis. Following RT-PCR, a melting curve analysis was performed to demonstrate the specificity of the PCR product as a single peak. A control, containing all the reaction components except for the template, was included in all experiments. All experiments were performed in triplicate.

Protein extraction. Cell lysates were prepared utilizing cell lysis buffer (Cell Signaling Technology, Danvers, Massachusetts) supplemented with Protease Arrest (G-Biosciences, St Louis, Missouri). Total protein concentration of cell lysates was measured using the Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, Illinois) according to the manufacturer's protocol.

Detection of GSR activity. The Glutathione Reductase Assay Kit (Cayman Chemical, Ann Arbor, Michigan) determines GSR activity by measuring the rate of NADPH oxidation. The oxidation of NADPH to NADP$^+$ is accompanied by a decrease in the absorbance at 340 nm. Since GSR is present at rate-limiting concentrations, the rate of decrease in the absorbance at 340 nm is
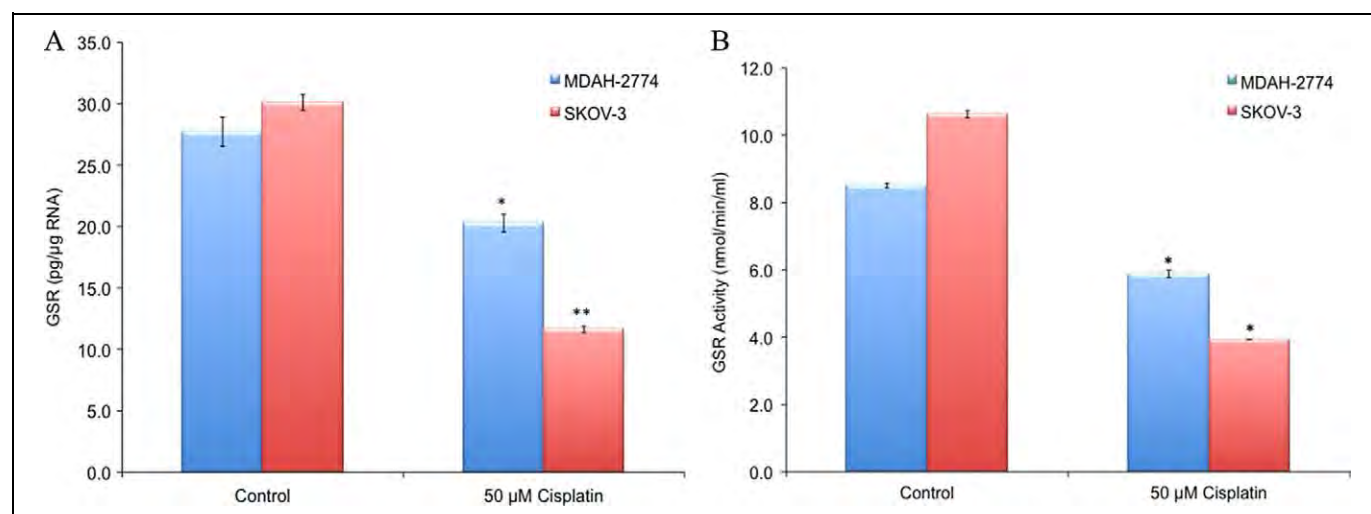
Figure 1. A, Real time RT PCR for GSR and (B) GSR activity in EOC cells. Sensitive (control) and resistant (50 μmol/L) cisplatin is correlated with GSR (A) mRNA levels and (B) activity. (*$P < .05$). EOC indicates epithelial ovarian cancer; GSR, glutathione reductase; mRNA, messenger RNA; RT PCR, real time reverse transcription polymerase chain reaction.

directly proportional to the GSR activity in the sample. The same concentration of protein was utilized for each sample.

Measurement of Nitrate/Nitrite Levels

The nitrate/nitrite colorimetric assay (Cayman Chemical, Ann Arbor, Michigan) was used to measure the levels of stable nitric oxide (NO) by-products, nitrate (NO_2), and nitrite (NO_3) as an indication of NO production. Due to the fact that the proportion of NO_2 and NO_3 is variable and cannot be predicted with certainty, the sum of both NO by-products is a more accurate indicator of NO production. The assay was performed utilizing cell culture media according to the manufacturer's protocol. Absorbance was detected at 540 nm, and a standard curve for nitrite was utilized to determine total NO_2 and NO_3 .

Measurement of Cellular Proliferation

Sensitive and cisplatin-resistant MDAH-2774 and SKOV-3 (5×10^3) EOC cells were cultured in 96-well tissue culture plates in 200 μL of medium (Becton Dickinson, Lincoln Park, New Jersey) and assessed for immunofluorescence as reported previously.[19] Nuclei expressing Ki-67 were labeled with a monoclonal antibody (Ki-S5; DAKO, Carpinteria, California). Briefly, after removing media from wells, the cells were fixed with 100 μL of formaldehyde for 30 minutes and then rinsed with phosphate-buffered saline (PBS). Subsequently, the cells were then treated with 100 μL of 0.01% Triton x-100 solution diluted in PBS. After administration of the primary antibody to wells and a 24-hour incubation period, the wells were washed 3 times with PBS, and then the FITC-conjugated secondary antibody was applied. Again, the wells were washed with PBS, and the nuclei were counterstained with 4′,6-diamidino-2-phenylindole. Finally, the wells were rinsed 3 times prior to visualization under the fluorescent microscope.

Statistical Analysis

Data were analyzed using SPSS 19.0. Unpaired *t* tests were used to compare controls and the resistant cell lines. *P* values are expressed at $\alpha < .05$ for significance.

Results

Cisplatin-Resistant EOC Cells Have Lower GSR mRNA and Activity Levels

There was a significant decrease in the mRNA expression and activity of GSR in both cisplatin-resistant EOC cell lines when compared to their sensitive counterparts (Figure 1A and B). The GSR mRNA levels decreased in cisplatin-resistant MDAH-2774 (20.3 ± 0.7 pg/μg RNA) and SKOV-3 (11.6 ± 0.25 pg/μg RNA) when compared to control MDAH-2774 (27.7 ± 1.2 pg/μg RNA) and control SKOV-3 (30.1 ± 0.6 pg/μg RNA) EOC cells ($P < .05$, Figure 1A).

Correspondingly, GSR protein activity also decreased in cisplatin-resistant MDH-2774 (from 8.5 ± 0.07 to 5.9 ± 0.1 nmol/min/mL) and SKOV-3 (from 10.6 ± 0.1 to 3.9 ± 0.0 nmol/min/mL) when compared to respective sensitive controls ($P < .05$, Figure 1B).

Cisplatin-Resistant EOC Cells Have Higher iNOS and Nitrate/Nitrite Levels

Nitrate/nitrite levels were significantly increased in SKOV-3 cisplatin-resistant EOC cells ($13.7 + 0.2$ μmol/L) when compared to its sensitive counterpart ($8.2 + 0.0$ μmol/L; $P < .05$, Figure 2A). There was no change observed in nitrate/nitrite levels in MDAH-2774-sensitive EOC cells when compared to its cisplatin-resistant counterpart.

The iNOS mRNA levels were significantly increased in MDAH-2774 cisplatin-resistant EOC cells (0.28 ± 0.01 fg/μg
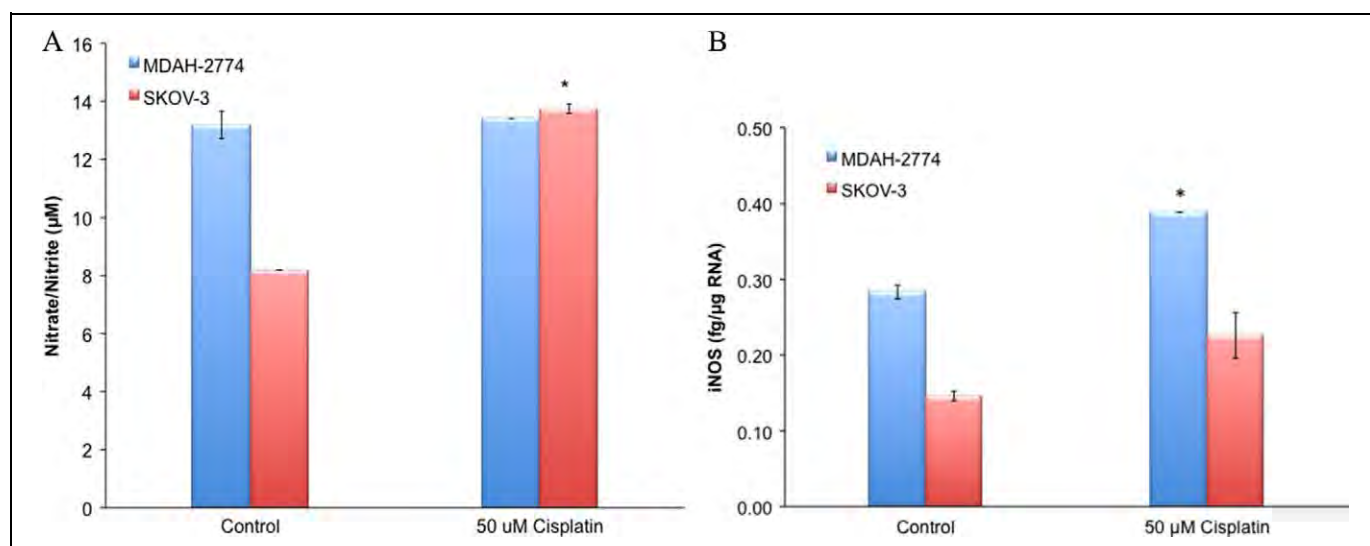
Figure 2. A, Real time RT PCR for iNOS and (B) nitrate/nitrate levels in EOC cells. Sensitive (control) and resistant (50 μmol/L) cisplatin is correlated with iNOS (A) mRNA levels and (B) nitrate/nitrite levels (*P < .05). EOC indicates epithelial ovarian cancer; iNOS, inducible nitric oxide synthase; mRNA, messenger RNA; RT PCR, real time reverse transcription polymerase chain reaction.
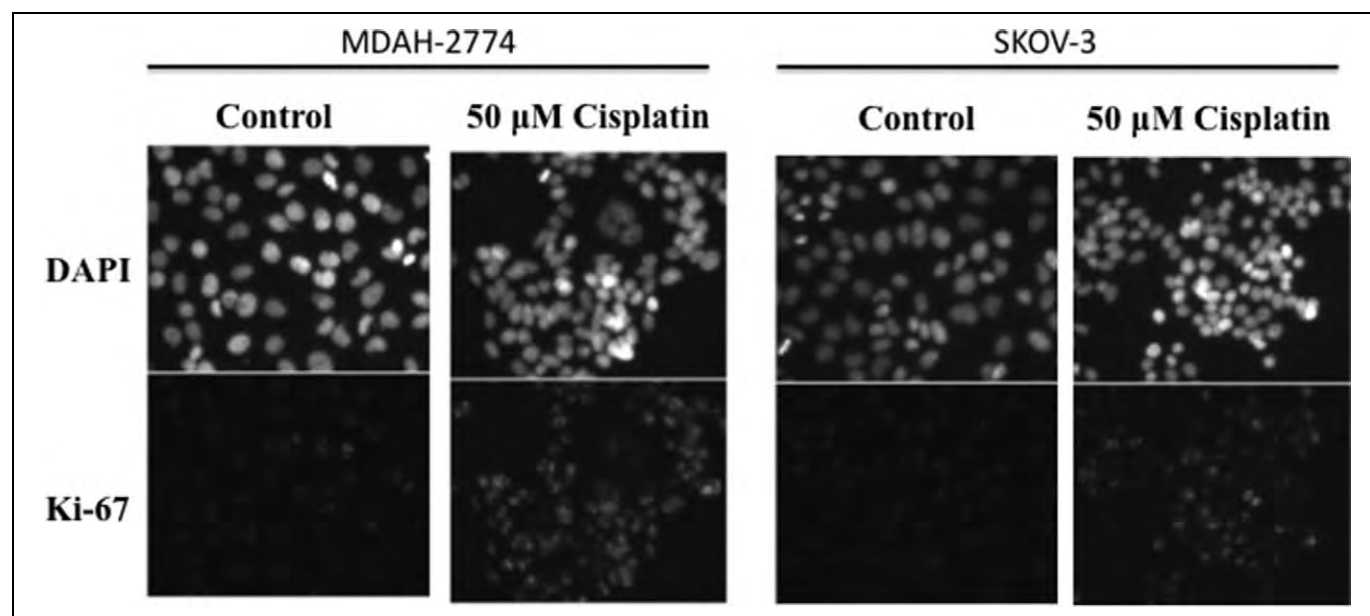

Figure 3. Proliferation of epithelial ovarian cancer cells lines (A) MDAH 2774 and (B) SKOV 3 cultured in the presence of cisplatin (50 μmol/L). The columns represent control and high dose (50 μmol/L) of cisplatin treatments and the rows illustrate anti Ki 67 antibody and 4′,6 diamidino 2 phenylindole counterstains.

RNA) when compared to its sensitive counterpart (0.39 ± 0.00 fg/μg RNA; $P < .05$, Figure 2B). There was an increased trend, however not significant, observed in iNOS mRNA levels in SKOV-3 when compared to its cisplatin-resistant counterpart.

Cisplatin-Resistant EOC Cells Have Increased Proliferation

The Ki-67 immunofluorescence staining revealed increased proliferation in cisplatin-resistant EOC cell lines when compared to their sensitive controls for both MDAH-2774 and SKOV-3 (Figure 3).

Discussion

In this study, we have shown that cisplatin-resistant EOC cells manifested a significant decrease in the mRNA expression and activity levels of GSR when compared to their sensitive counterparts (Figure 1A and B). Glutathione (GSH), a tripeptide, is a powerful antioxidant that plays an important

role in preventing ROS-induced damages to vital cellular functions.[20-22] However, it was shown that once cancer is established, high levels of GSH could be deleterious by preventing the cytotoxic effect of various chemotherapeutic agents through detoxification and increased activity of efflux pump.[23-25] Conversely, it has also been reported that low levels of GSH are linked to impaired immune response and tumor progression.[26-28] The latter is more aligned with our findings, because low expression of GSR is indicative of low GSH and increased oxidative stress, since GSH will not be adequately regenerated from its oxidized form (GSSG). Together, the antioxidant mechanisms involving GSR among others, when impaired, may adversely impact the response to cisplatin by failing to restore the oxidative balance.

On the other hand, cisplatin-resistant MDAH-2774 EOC cells manifested an increase in iNOS expression when compared to their sensitive counterpart. Moreover, nitrate/nitrite levels were significantly increased in cisplatin-resistant SKOV-3 EOC cells when compared to its sensitive counterparts. Our results suggest that increased expression of iNOS and nitrate/nitrite may be associated with resistance to cisplatin. The NO synthase family includes the calcium/calmodulin-mediated isoenzymes endothelial NOS (eNOS) and neuronal NOS (nNOS), in addition to the noncalcium-dependent iNOS.[29] Earlier studies have shown that NO donors can induce apoptosis at high concentrations, whereas preventing it can be achieved at low physiologic levels.[30,31] Moreover, when compared to normal cells, cancer cells have significantly increased level of iNOS.[32] Previously, we have reported that silencing iNOS can lead to a decrease in S-nitrosylation of caspase-3 with subsequent increased apoptosis in EOC cells.[14] Also, we have demonstrated that high levels of iNOS in EOC cells are associated with high levels of vascular endothelial growth factor (VEGF) production and angiogenesis induction.[6] Additionally, other groups have reported an inverse relationship between the expression of eNOS/nNOS and iNOS that suggests elevated expression of iNOS coupled with decreased eNOS/nNOS expression may be associated with p53-mediated cisplatin resistance in EOC.[33] Conversely, low expression of iNOS in head and neck squamous cell carcinoma was shown to be associated with resistance to cisplatin/taxol-induced apoptosis mediated through survivin.[34] The physiology of the NO synthase family is fairly complex and not fully elucidated; however, they appeared to be involved in cisplatin resistance in many cancers.

Finally, utilizing Ki-67, we observed a significant increase in proliferation of cisplatin-resistant EOC cells when compared to their sensitive counterparts.

Several possible mechanisms of cisplatin resistance have been previously described, including decreased intracellular aquation, decreased uptake, and accelerated efflux due to increased activity of adenosine triphosphate (ATP)-binding cassette and soluble carrier transporter families.[35-37] Additionally, poor tissue perfusion, increased proliferation, and decreased apoptosis altogether contribute to chemoresistance. The present study suggests a potential role for oxidative stress in the development of cisplatin resistance in ovarian cancer.

Understanding the precise mechanism by which oxidative stress contributes to the development of cisplatin resistance will significantly impact survival of patients with ovarian cancer.

Authors' Note

Jimmy Belotte and Nicole M. Fletcher contributed equally to this work.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

References

1. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2013. *CA Cancer J Clin*. 2013;63(1):11 30.
2. Markman M, Rothman R, Hakes T, et al. Second line platinum therapy in patients with ovarian cancer previously treated with cisplatin. *J Clin Oncol*. 1991;9(3):389 393.
3. Diplock AT, Rice Evans CA, Burdon RH. Is there a significant role for lipid peroxidation in the causation of malignancy and for antioxidants in cancer prevention? *Cancer Res*. 1994;54(7 suppl):1952s 1956s.
4. Kamata H, Hirata H. Redox regulation of cellular signalling. *Cell Signal*. 1999;11(1):1 14.
5. Jiang Z, Fletcher NM, Ali Fehmi R, et al. Modulation of redox signaling promotes apoptosis in epithelial ovarian cancer cells. *Gynecol Oncol*. 2011;122(2):418 423.
6. Malone JM, Saed GM, Diamond MP, Sokol RJ, Munkarah AR. The effects of the inhibition of inducible nitric oxide synthase on angiogenesis of epithelial ovarian cancer. *Am J Obstet Gyne col*. 2006;194(4):1110 1116; discussion 1116 1118.
7. Dvoriantchikova G, Grant J, Santos AR, Hernandez E, Ivanov D. Neuronal NAD(P)H oxidases contribute to ROS production and mediate RGC death after ischemia. *Invest Ophthalmol Vis Sci*. 2012;53(6):2823 2830.
8. Montezano AC, Touyz RM. Molecular mechanisms of hypertension reactive oxygen species and antioxidants: a basic sci ence update for the clinician. *Can J Cardiol*. 2012;28(3):288 295.
9. Valko M, Morris H, Cronin MT. Metals, toxicity and oxidative stress. *Curr Med Chem*. 2005;12(10):1161 1208.
10. Evans MD, Cooke MS. Factors contributing to the outcome of oxi dative damage to nucleic acids. *Bioessays*. 2004;26(5):533 542.
11. Halliwell B. Oxidative stress and cancer: have we moved for ward? *Biochem J*. 2007;401(1):1 11.
12. Warburg O. On the origin of cancer cells. *Science*. 1956;123(3191):309 314.
13. Lopez Lazaro M. The warburg effect: why and how do cancer cells activate glycolysis in the presence of oxygen? *Anticancer Agents Med Chem*. 2008;8(3):305 312.

14. Saed GM, Ali Fehmi R, Jiang ZL, et al. Myeloperoxidase serves as a redox switch that regulates apoptosis in epithelial ovarian cancer. *Gynecol Oncol*. 2010;116(2):276 281.

15. Fletcher NM, Jiang Z, Ali Fehmi R, et al. Myeloperoxidase and free iron levels: potential biomarkers for early detection and prog nosis of ovarian cancer. *Cancer Biomark*. 2011;10(6):267 275.

16. Akimaru K, Kuo MT, Furuta K, Suzuki M, Noyori R, Ishikawa T. Induction of MRP/GS X pump and cellular resistance to antican cer prostaglandins. *Cytotechnology*. 1996;19(3):221 227.

17. Fujii R, Mutoh M, Sumizawa T, Chen ZS, Yoshimura A, Akiyama S. Adenosine triphosphate dependent transport of leu kotriene C4 by membrane vesicles prepared from cisplatin resistant human epidermoid carcinoma tumor cells. *J Natl Cancer Inst*. 1994;86(23):1781 1784.

18. Ishikawa T, Ali Osman F. Glutathione associated cis diammi nedichloroplatinum(II) metabolism and ATP dependent efflux from leukemia cells. Molecular characterization of glutathione platinum complex and its biological significance. *J Biol Chem*. 1993;268(27):20116 20125.

19. Armant DR, Kilburn BA, Petkova A, et al. Human trophoblast sur vival at low oxygen concentrations requires metalloproteinase mediated shedding of heparin binding EGF like growth factor. *Development*. 2006;133(4):751 759.

20. Han YH, Moon HJ, You BR, Kim SZ, Kim SH, Park WH. The effects of N acetyl cysteine on the MG132 proteasome inhibitor treated lung cancer cells in relation to cell growth, reactive oxygen species and glutathione. *Int J Mol Med*. 2010;25(4):657 662.

21. Chow HH, Hakim IA, Vining DR, et al. Modulation of human glutathione s transferases by polyphenon e intervention. *Cancer Epidemiol Biomarkers Prev*. 2007;16(8):1662 1666.

22. Balendiran GK, Dabur R, Fraser D. The role of glutathione in can cer. *Cell Biochem Funct*. 2004;22(6):343 352.

23. Batist G, Behrens BC, Makuch R, et al. Serial determinations of glu tathione levels and glutathione related enzyme activities in human tumor cells in vitro. *Biochem Pharmacol*. 1986;35(13):2257 2259.

24. Suzuki T, Nishio K, Tanabe S. The MRP family and anticancer drug metabolism. *Curr Drug Metab*. 2001;2(4):367 377.

25. de Bittencourt Junior PI, Curi R, Williams JF. Glutathione meta bolism and glutathione S conjugate export ATPase (MRP1/GS X pump) activity in cancer. I. Differential expression in human can cer cell lines. *Biochem Mol Biol Int*. 1998;45(6):1227 1241.

26. Suthanthiran M, Anderson ME, Sharma VK, Meister A. Glu tathione regulates activation dependent DNA synthesis in highly purified normal human T lymphocytes stimulated via the CD2 and CD3 antigens. *Proc Natl Acad Sci USA*. 1990;87(9): 3343 3347.

27. Multhoff G, Meier T, Botzler C, et al. Differential effects of ifos famide on the capacity of cytotoxic T lymphocytes and natural killer cells to lyse their target cells correlate with intracellular glu tathione levels. *Blood*. 1995;85(8):2124 2131.

28. Droge W, Schulze Osthoff K, Mihm S, et al. Functions of glu tathione and glutathione disulfide in immunology and immuno pathology. *FASEB J*. 1994;8(14):1131 1138.

29. Forstermann U, Gath I, Schwarz P, Closs EI, Kleinert H. Iso forms of nitric oxide synthase. Properties, cellular distribution and expressional control. *Biochem Pharmacol*. 1995;50(9): 1321 1332.

30. Fiscus RR. Involvement of cyclic GMP and protein kinase G in the regulation of apoptosis and survival in neural cells. *Neuro signals*. 2002;11(4):175 190.

31. Fiscus RR, Yuen JP, Chan SL, Kwong JH, Chew SB. Nitric oxide and cyclic GMP as pro and anti apoptotic agents. *J Card Surg*. 2002;17(4):336 339.

32. Wink DA, Vodovotz Y, Cook JA, et al. The role of nitric oxide chemistry in cancer treatment. *Biochemistry (Mosc)*. 1998; 63(7):802 809.

33. Leung EL, Fraser M, Fiscus RR, Tsang BK. Cisplatin alters nitric oxide synthase levels in human ovarian cancer cells: involvement in p53 regulation and cisplatin resistance. *Br J Cancer*. 2008; 98(11):1803 1809.

34. Fetz V, Bier C, Habtemichael N, et al. Inducible NO synthase confers chemoresistance in head and neck cancer by modulating survivin. *Int J Cancer*. 2009;124(9):2033 2041.

35. Liu J, Cristea MC, Frankel P, et al. Clinical characteristics and outcomes of BRCA associated ovarian cancer: genotype and sur vival. *Cancer Genet*. 2012;205(1 2):34 41.

36. Ali AY, Farrand L, Kim JY, et al. Molecular determinants of ovarian cancer chemoresistance: new insights into an old conun drum. *Ann N Y Acad Sci*. 2012;1271:58 67.

37. Kalinina EV, Berezov TT, Shtil AA, et al. Expression of peroxir edoxin 1, 2, 3, and 6 genes in cancer cells during drug resistance formation. *Bull Exp Biol Med*. 2012;153(6):878 881.

Exhibit 85

267

Myeloperoxidase and free iron levels: Potential biomarkers for early detection and prognosis of ovarian cancer

Nicole M. Fletcher[a], Zhongliang Jiang[a], Rouba Ali-Fehmi[b], Nancy K. Levin[c], Jimmy Belotte[a], Michael A. Tainsky[c], Michael P. Diamond[a], Husam M. Abu-Soud[a] and Ghassan M. Saed[a,*]

[a]*Department of Obstetrics and Gynecology, The C.S. Mott Center for Human Growth and Development, Wayne State University School of Medicine, Detroit, MI, USA*

[b]*Department of Pathology, Wayne State University School of Medicine, Detroit, MI, USA*

[c]*Department of Oncology, Karmanos Cancer Institute, Detroit, MI, USA*

Abstract. *Objective:* The study sought to identify whether a relationship exists between serum myeloperoxidase (MPO) and free iron with stages of ovarian cancer.

Methods: Serum and tissue samples were collected from women with stages I through IV ovarian cancer, benign gynecologic conditions, inflammation, and healthy controls. Myeloperoxidase ELISA and VITROS Fe Slide assays were used to measure serum and tissue MPO and free iron levels, respectively. Data were analyzed with a one-way ANOVA with post-hoc comparisons ($p < 0.05$ considered significant).

Results: There was a significant increase in the level of free iron in serum and tissues obtained from stages II–IV as compared to early-stage (stage I) ovarian cancer. There was an overlap between early-stage and inflammation serum MPO levels, however serum free iron levels were significantly higher in early-stage. There was no significant change in serum free iron levels between non-cancer groups. In contrast, there was a significant increase in serum free iron levels in early-stage as compared to non-cancer groups.

Conclusions: Collectively, these findings clearly indicate a role for the combination of serum MPO and free iron as biomarkers for early detection and prognosis of ovarian cancer.

Keywords: Myeloperoxidase, free iron, ovarian cancer, biomarkers

1. Introduction

Ovarian cancer is the fifth leading cause of cancer death in women in the United States, the leading cause of death from gynecologic malignancies, and the second most commonly diagnosed gynecologic malignancy; yet the underlying pathophysiology is not clearly understood [1]. Because early-stage ovarian cancer presents with nonspecific symptoms, diagnosis is often not made until after the malignancy has spread beyond the ovaries [2]. Mortality rates for this type of malignancy are high due to lack of early-stage screening methods [2]. While the 10-year survival rate for localized ovarian cancer is approximately 90%, it drops to about 60% for regional disease and about 20% for women with metastatic disease. Routine screening for ovarian cancer in the general population is not recommended because traditional screening methods are neither sensitive nor specific enough [2,3]. Therefore, the development of sensitive and specific methods for early detection has been a priority as a means for improving the diagnosis and treatment of this disease.

*Corresponding author: Ghassan M. Saed, Ph.D., Department of Obstetrics and Gynecology, C.S. Mott Center for Human Growth and Development, Wayne State University School of Medicine, 275 E. Hancock, Detroit, MI 48201, USA. Tel.: +1 313 577 5433; Fax: +1 313 577 4633; E-mail: gsaed@med.wayne.edu.

Clinical studies have documented the association between inflammation and certain cancers for decades [4]. There is convincing evidence that inflammation is a contributing factor in ovarian cancer development, but the role of complement-induced inflammation in tumor initiation or progression remains poorly investigated [5, 6]. Stimulated inflammatory cells are capable of inducing genotoxic effects, such as DNA strand breaks, sister chromatid exchanges and mutations, and promotion of neoplastic transformation in nearby cells [7]. Myeloperoxidase (MPO) is an enzyme stored in the azurophilic granules of polymorphonuclear neutrophils and macrophages, and is released into the extracellular fluid in response to inflammation. Myeloperoxidase utilizes hydrogen peroxide (H_2O_2), the dismutation product of superoxide ($O_2^{\bullet-}$), to generate hypochlorous acid (HOCl), a cytotoxic and antimicrobicidal oxidant. The role of MPO in carcinogenesis has been implicated in both the activation of procarcinogens to genetoxic intermediates and the potentiation of xenobiotic carcinogenicity [8,9].

We have recently reported that MPO is expressed in epithelial ovarian cancer (EOC) cells and tissues with limited or no expression in normal ovarian tissues [10]. Studies from our laboratory clearly indicated that MPO is responsible for the S-nitrosylation of caspase-3, which inhibited its activity [10]. Silencing MPO gene expression by the utilization of MPO specific siRNA induced apoptosis in EOC cells through a mechanism that decreases MPO-induced S-nitrosylation of caspase-3 [10]. Additionally, we have compelling evidence that leads us to believe that MPO may serve as a source of free iron under oxidative stress, where both NO and $O_2^{\bullet-}$ are elevated [11]. Recently we have shown that, HOCl, the final product of MPO, displays the potential capacity to mediate hemoprotein heme destruction and subsequent iron release, providing a potential link between elevated MPO and free iron [12–14].

The free iron generated by hemeoprotein destruction not only contributes to elevation of serum iron levels, but may also induce oxidative stress, which can promote lipid peroxidation, DNA strand breaks, and modification or degradation of biomolecules [15–17]. Iron reacts with H_2O_2 and catalyzes the generation of highly reactive hydroxyl radicals, which in turn further increases free iron concentrations by the Fenton and Haber–Weiss reaction [18]. Several studies from our laboratories have provided a mechanistic link between oxidative stress, MPO, higher levels of HOCl and higher free iron that could explain the observed accumulation of free iron in epithelial ovarian cancers tissues [10–14].

The search for non-invasive, cost-effective ovarian cancer biomarker tests has been ongoing for many years. Immunizations of mice with ovarian cancer cells has led to hybridoma validation by ELISA, while flow cytometry analysis permitted the discovery of cancer antigen (CA)-125 and mesothelin [19]. Furthermore, the screening of an array of 21,500 unknown ovarian cDNAs hybridized with labeled first-strand cDNA from ten ovarian tumors and six normal tissues led to the discovery of human epididymis protein 4 (HE4) [20]. Most interestingly, HE4 is overexpressed in 93% of serous and 100% of endometrioid EOCs, and in 50% of clear cell carcinomas, but not in mucinous ovarian carcinomas [21]. Although it is not tissue-specific, a number of independent microarray studies identified HE4 as one of the most useful biomarkers for ovarian cancer [20,22–24]. In addition to expression on the cellular level, secreted HE4 was detected in high levels in the serum of ovarian cancer patients [25]. Additionally, combining CA-125 and HE4 is a more accurate predictor of malignancy than either alone [26–28].

In this study, we sought to identify a positive correlation between serum MPO and free iron with stage of ovarian cancer as well as tissue MPO and free iron with stage of ovarian cancer.

2. Material and methods

2.1. Patient population

A description of the demographics of the study groups are listed in Tables 1 and 2.

2.1.1. Ovarian cancer cases

Sera ($N = 15$) and tissues ($N = 27$) were collected from patients presenting to the gynecologic oncology division of Karmanos Cancer Institute with suspected EOC were invited to participate in a prior study. They underwent informed consent (Wayne State University Human Subject Committee protocol number 027201MP2E) and agreed to provide a blood sample prior to treatment (chemotherapy or surgery). Cases include early through late-stage diagnoses as well as a variety of histologies. Stage I is designated as early-stage, as compared to remaining stages II through IV (II–IV).

2.1.2. Benign controls

Sera ($N = 14$) and tissues ($N = 14$) from patients with benign gynecologic conditions was procured through the Cooperative Human Tissue Network

Table 1
Descriptive statistics for age including Mean, Median, and Standard deviation. $N = 30$

			Status		p-value < 0.05
			No cancer	Ovarian cancer	Sig. (2-sided)
Race	Black	Count	5	1	0.131
		% within race:	83.3%	16.7%	
		% within status:	33.3%	6.7%	
	Other	Count	0	1	
		% within race:	0%	100%	
		% within status:	0%	100%	
	White	Count	10	13	
		% within race:	43.5%	56.5%	
		% within status:	66.7%	86.7%	
Age	LE median (53)	Count	10	6	0.272
		% within age:	62.5%	37.5%	
		% within status:	66.7%	40.0%	
	GT median (53)	Count	5	9	
		% within age:	35.7%	64.3%	
		% within status:	33.3%	60.0%	
Region/Source	CHTN	Count	6	0	0.000
		% within region/source:	100%	0%	
		% within status:	40.0%	0%	
	GOG	Count	0	11	
		% within region/source:	0%	100%	
		% within status:	0%	73.3%	
	KCI	Count	8	2	
		% within region/source:	80.0%	20.0%	
		% within status:	53.3%	13.3%	
	Mayo Clinic	Count	1	2	
		% within region/source:	33.3%	66.7%	
		% within status:	6.7%	13.3%	
Total			15	15	
			50%	50%	
			100%	100%	

Table 2
Comparative study of the cases and control based on cancer status. Pearson Chi-Square was used for statistical test, $p < 0.05$ was considered statistically significant

Descriptives				
			Statistic	Std. error
Age at Dx/Entry into study:	Mean		52.47	1.947
	95% Confidence interval for mean	Lower bound	48.48	
		Upper bound	56.45	
	5% Trimmed mean		52.43	
	Median		53.00	
	Variance		113.706	
	Std. Deviation		10.663	
	Minimum		24	
	Maximum		78	
	Range		54	
	Interquartile range		10	
	Skewness		−0.030	0.427
	Kurtosis		1.832	0.833

There was no statistically difference between the two groups based on age and race. However, the region or source where the specimen was obtained demonstrated a statistically significant difference that can be attributed to the regional and centers characteristics.

(CHTN). These include women diagnosed with ovarian cysts, endometriosis (inflammation), or uterine fibroids.

2.1.3. Healthy controls

Healthy control sera ($N = 8$) were procured from women recruited through a local community organization. These women presented as healthy, with no history of cancer. Basic information such as age, race, and evidence of benign gynecologic conditions, if any, was obtained at the time of informed consent. The age and racial makeup of this group overlaps with patients with ovarian cancer and benign conditions but is not explicitly matched.

Additionally, we utilized matched tissue samples from the same patient derived serum samples to assess the correlation between MPO and free iron in these tissues. In this way we assessed if there was an expected discrimination in the tissues and to what extent that discrimination diluted in the serum.

2.2. Local samples were processed as follows

A single 7 cc vial of blood was obtained during normal phlebotomy and the serum was isolated after clotting. Serum was collected into a red top or SST blood tube. The serum clot was allowed to form over 1–2 hours and was pelleted in a desktop centrifuge at 2400 rpm for 10 min. The serum was removed by pipetting and ~1 ml aliquots were stored at $-80°C$. Samples were labeled with a coded identifier.

2.3. Detection of Myeloperoxidase

Myeloperoxidase was detected with the Myeloperoxidase Enzyme Immunometric Assay Kit (Enzo Life Sciences, Farmingdale, NY). This is a well-established assay in our laboratory and was performed according to the manufacturer's protocol. This kit is for the quantitative determination of MPO in biological fluids or tissues. The kit uses a monoclonal antibody to MPO immobilized on a microtiter plate to bind the MPO in the standards or sample. The measured optical density (450 nm) is directly proportional to the concentration of MPO. The sensitivity of the assay, defined as the concentration of human MPO was determined to be 0.019 ng/mL. All experiments were performed in triplicate.

2.4. Detection of free iron

The VITROS Fe Slide method (VITROS 750, Johnson & Johnson, Rochester, USA) was used for detecting serum and tissue free iron levels as described by the manufacturer's protocol. Briefly, 10 μL of sample is applied to a VITROS Fe DT Slide, which then is loaded into a VITROS 950 chemistry system (Ortho Clinical Diagnostics, Inc) located at the University Health Building. The iron in the sample was removed from transferrin at acidic pH and migrates to a reducing layer where ascorbic acid reduces it to the ferrous form. The ferrous iron then is bound to a dye, producing color that is detected (600 nm) as a rate of change in reflection density. This assay has a reportable range of 10 to 500 μg/dL, and is therefore is suitable for detection in both tissues and sera. All experiments were performed in triplicate.

2.5. Statistical analysis

Data were analyzed using SPSS 19.0 for windows (SPSS for Windows, Chicago, IL). Data were analyzed using oneway ANOVA (analysis of variance) with Student Neuman-Kuels post-hoc comparisons. Significance values of $p < 0.05$ were considered statistically significant for all analyses.

3. Results

Ovarian cancer stages II–IV manifested higher levels of tissue MPO. All stages of ovarian cancer had significantly higher levels of MPO as compared to benign and inflammatory groups (Fig. 1, $p < 0.05$). Also, ovarian cancer stages II–IV had a significantly higher level of MPO as compared to early-stage ovarian cancer (Fig. 1, $p < 0.05$). The benign group is not significantly different from the inflammatory group. At the time of running this experiment, no healthy control tissue samples were available.

Ovarian cancer stages II–IV manifested higher levels of serum MPO. Ovarian cancer stages II–IV had significantly higher levels of MPO as compared to early-stage ovarian cancer, control, benign, and inflammatory groups ($p < 0.05$, Fig. 2). Early-stage ovarian cancer and inflammatory gynecologic conditions are significantly different from the control and benign groups, while not significantly different from one another ($p < 0.05$, Fig. 2). Also, control and benign groups were not significantly different.

Ovarian cancer stages II–IV manifested higher levels of tissue free iron. All stages of ovarian cancer had significantly higher levels of tissue free iron than benign and inflammatory groups ($p < 0.05$, Fig. 3). In

N.M. Fletcher et al. / Potential biomarkers for early detection and prognosis of ovarian cancer 271
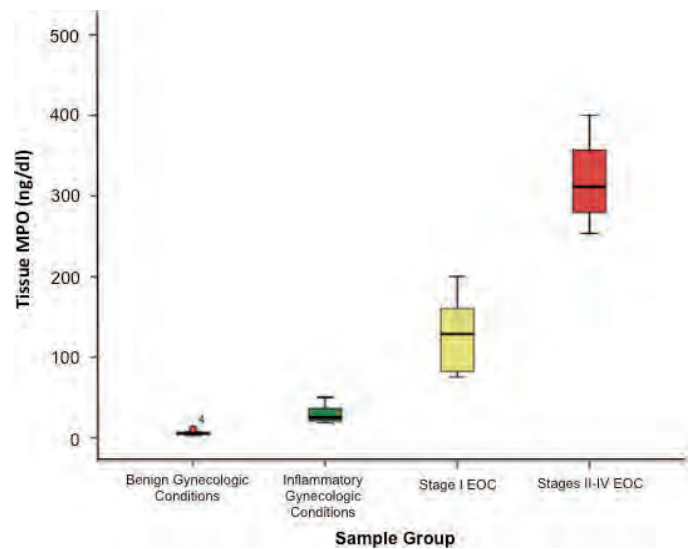

Fig. 1. Tissue MPO: Serum and tissue MPO was detected with the Myeloperoxidase Enzyme Immunometric Assay Kit, as described in methods. Early-stage ovarian cancer ($n = 5$) and ovarian cancer stages II–IV ($n = 22$) are significantly different than all other groups and each other ($p < 0.05$). The benign group ($n = 14$) is not significantly different from the inflammatory group ($n = 7$). At the time of running this experiment, no healthy control tissue samples were available. In benign gynecologic conditions, the outlier represents a value more than three interquartile ranges above the 75th percentile value.
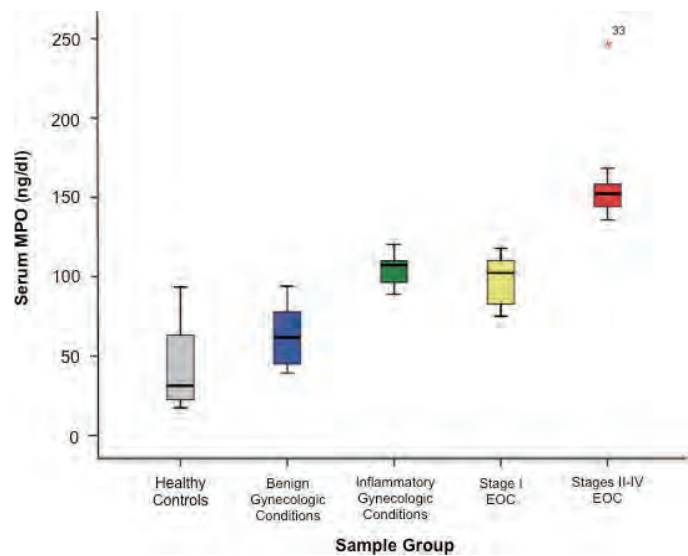

Fig. 2. Serum MPO: Serum and tissue MPO was detected with the Myeloperoxidase Enzyme Immunometric Assay Kit, as described in methods. Ovarian cancer stages II–IV ($n = 10$) are significantly different than all other groups ($p < 0.05$). Early-stage ovarian cancer ($n = 5$) and inflammatory gynecologic conditions ($n = 7$) are different from control ($n = 8$) and benign groups ($n = 14$), while not one another ($p < 0.05$). Control and benign groups are not statistically different. In stages II–IV, the outlier represents a value more than three interquartile ranges above the 75th percentile value.

addition, ovarian cancer stages II–IV had higher levels of free iron as compared to early-stage ovarian cancer ($p < 0.05$, Fig. 3). The benign group is not significantly different from the inflammatory group. At the time of running this experiment, no healthy control tissue samples were available.

Ovarian cancer stages II–IV manifested higher levels of serum free iron All stages of ovarian cancer had significantly higher levels of serum free iron as compared to control, benign, and inflammatory groups ($p <$
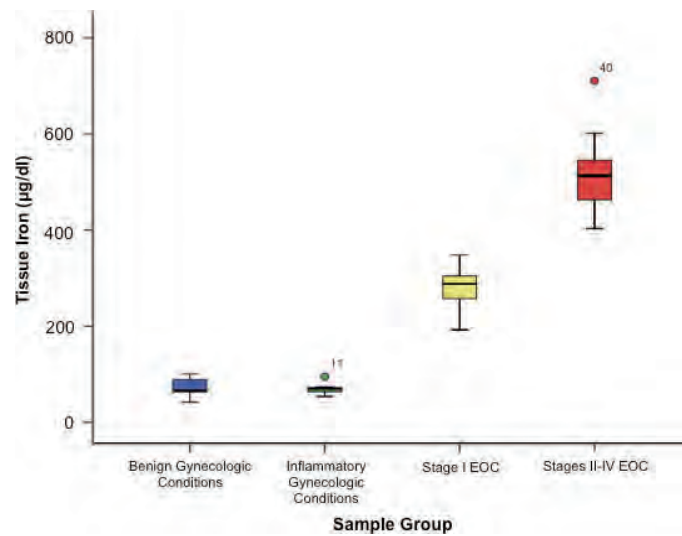
Fig. 3. Tissue free iron: Serum and tissue free iron were detected using the VITROS Fe Slide method as described in methods. Ovarian cancer stages II–IV ($n = 22$) and early-stage ovarian cancer ($n = 5$) are significantly different than all other groups as well as each other ($p < 0.05$). The benign group ($n = 14$) is not significantly different from the inflammatory group ($n = 7$). At the time of running this experiment, no healthy control tissue samples were available. In inflammatory gynecologic conditions and stages II–IV, the outliers represent a value more than three interquartile ranges above the 75th percentile value.

0.05, Fig. 4). In addition, ovarian cancer stages II–IV had higher serum free iron levels as compared to early-stage ovarian cancer ($p < 0.05$, Fig. 4). There are no significant differences between control, benign and inflammatory groups.

4. Discussion

The findings from this study indicate a role for the combination of serum MPO and free iron as biomarkers for early detection and prognosis of ovarian cancer. Multi-marker panels have the potential for high positive predictive values (PPVs), but careful validation with appropriate sample cohorts is mandatory and complex algorithms may be difficult to implement for routine clinical use [19]. Panels of biomarkers have been extensively investigated to improve sensitivity and specificity and have included some of the most promising reported markers such as CA72-4, M-CSF, OVX1, LPA, Prostacin, Osteopontin, Inhibin and Kallikrein [29–31]. While these and other potential screening paradigms with comparable sensitivities and specificities have been previously reported, these assays often require specialized equipment not routinely utilized in the clinical immunoassay laboratory, or rely on complex computational algorithms to generate adequate assay performance [32]. With an ovarian cancer prevalence of only 1 in 2500 among postmenopausal

women in the U.S., an effective screening strategy for the general population needs to attain a sensitivity of 75% and specificity about 99.6% to attain a minimally acceptable potential PPV of 10% for the detection of all stages of ovarian cancer [32]. No single biomarker reported to date has met these thresholds.

The observation of the involvement of MPO in oxidative stress and inflammation has been a leading factor in the study of MPO as a possible marker of plaque instability and a useful clinical tool in the evaluation of patients with coronary heart disease [33]. Recent genetic studies implicated MPO in the development of lung cancer by demonstrating a striking correlation between the relative risk for development of the disease and the incidence of functionally distinct MPO polymorphisms [34].

We now have substantial evidence to believe that the presence of MPO may play a role in maintaining the oncogenic phenotype of EOC cells [10]. In support of this notion, polymorphisms at position -463 (G to A) in the promoter region of the MPO gene have been identified, with the A variant allele related to decreased transcriptional activity and decreased MPO expression, which may be associated with reduced risk of human cancers [35]. Myeloperoxidase requires heme as a cofactor, which consists of an iron atom contained in the center of a large heterocyclic organic ring [36]. Thus, the MPO G allele might produce more reactive oxygen species when iron intake is high. Previous studies have
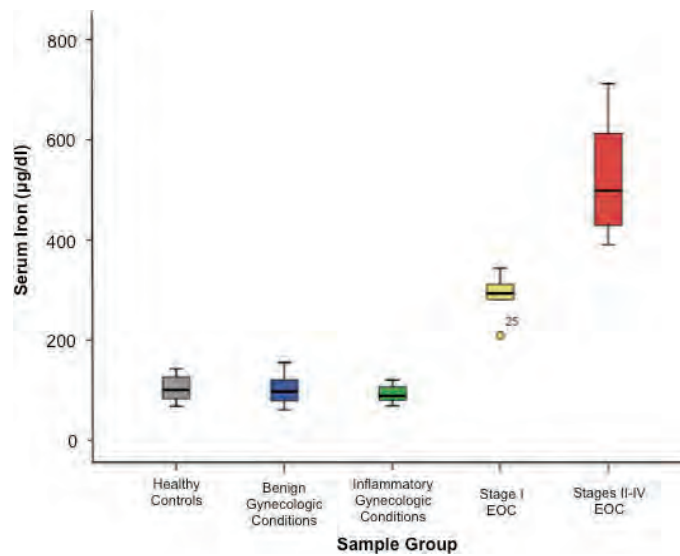
Fig. 4. Serum free iron: Serum and tissue free iron were detected using the VITROS Fe Slide method as described in methods. Ovarian cancer stages II–IV ($n = 10$) and early-stage ovarian cancer ($n = 5$) are significantly different than other groups as well as each other ($p < 0.05$). There are no significant differences among the control ($n = 8$), benign ($n = 14$) and inflammatory groups ($n = 7$). In stage I, the outlier represents a value more than three interquartile ranges above the 75th percentile value.

investigated the combined roles of MPO and iron levels on cancer development, as well as assessed the impact of MPO genetic polymorphisms on the development of cirrhosis with hereditary hemochromatosis [37, 38]. They found that the MPO GG genotype was more common in patients with cirrhosis than in those without, modifying the clinical penetrance of hepatic iron overload with respect to hepatic fibrosis in hereditary hemochromatosis.

Myeloperoxidase levels reported for various inflammatory disorders are coincidentally lower than those levels found in all stages of ovarian cancer. A previous study reported normal serum MPO and iron levels as 62 ± 11 ng/ml and 96 ± 9 µg/dl, respectively [39]. However, there was a significant increase in serum MPO and iron levels to 95 ± 20 ng/ml, and 159 ± 20 µg/dl, respectively, in asthmatic individuals [39]. Although there was an increase in this reported serum iron, these levels still fell within the normal range (50 to 170 µg/dl) [40,41]. Other studies have showed that an elevated MPO level, reaching up to 350 ng/ml, in serum plasma, was indicative of a higher risk for cardiovascular events in patients hospitalized for chest pain [42,43]. Our data showed an overlap between serum MPO levels in early-stage ovarian cancer with inflammation. However, serum free iron levels were significantly higher in early-stage ovarian cancer as compared to inflammation. Thus, there is a potential for a false positive with

MPO alone in patients with cardiovascular, inflammation, and/or asthmatic disorders.

Utilizing serum free iron levels alone as a biomarker is also not sufficient for early detection of ovarian cancer due to many uncontrolled variables, i.e. dietary intake, supplements, effects of other iron-generating enzymes or factors, and more importantly they are not as specific as MPO levels. Specifically, in iron deficiency anemic patients, their free iron levels may become a confounding factor in its utilization for early detection of ovarian cancer. Thus, anemia should be ruled out to eliminate any overlap that would lead to misdiagnosis. The incorporation of iron deficiency anemic patients in a logistic regression model will help determine its overlap with early-stage ovarian cancer. Additionally, currently available clinical studies focused on either biochemical or more recently, genetic markers of iron overload have reported conflicting results regarding the use of free iron levels alone for diagnosis [44–47]. For these reasons, we expect that the combination of serum MPO and free iron levels to yield a higher power of specificity and sensitivity that should distinguish women with early-stage ovarian cancer from other disorders, specifically inflammation. Additionally, combining serum MPO and free iron levels with the best currently existing biomarkers through the creation of a logistic regression model may increase the overall predictive values. Collectively, our data strongly supports a role for serum MPO and free iron in the pathophysiol-

ogy of ovarian cancer, which thereby qualifies them to serve as biomarkers for early detection and prognosis of ovarian cancer.

To validate MPO and free iron as biomarkers for early detection of ovarian cancer, we are currently planning a follow up study on a larger population with the principal endpoint of establishing the best cutoff point using the receiver operating characteristic (ROC) method [48–50]. Also, we will generate sensitivity, specificity, and positive and negative predictive values for serum MPO and free iron alone and in combination. These results will be compared to the existing CA-125 biomarker. As a secondary endpoint, we plan to performed survival analysis using the Kaplan-Meier method with MPO and free serum levels as factors.

References

[1] Jemal, A., et al., Cancer statistics, 2006. CA Cancer J Clin, 2006. **56**(2): pp. 106-30.

[2] Leahy, Y., Are serum protein biomarkers effective in detecting ovarian cancer in its early stages? Clin J Oncol Nurs, 2009. **13**(4): pp. 443-5.

[3] Screening for ovarian cancer: recommendation statement. U.S. Preventive Services Task Force. Am Fam Physician, 2005. **71**(4): pp. 759-62.

[4] Balkwill, F. and A. Mantovani, Inflammation and cancer: back to Virchow? Lancet, 2001. **357**(9255): pp. 539-45.

[5] Ness, R.B. and C. Cottreau, Possible role of ovarian epithelial inflammation in ovarian cancer. J Natl Cancer Inst, 1999. **91**(17): pp. 1459-67.

[6] Markiewski, M.M., et al., Modulation of the antitumor immune response by complement. Nat Immunol, 2008. **9**(11): pp. 1225-35.

[7] Rosin, M.P., Anwar, W.A. and A.J. Ward, Inflammation, chromosomal instability, and cancer: the schistosomiasis model. Cancer Res, 1994. **54**(7 Suppl): pp. 1929s-1933s.

[8] Podrez, E.A., et al., Myeloperoxidase-generated reactive nitrogen species convert LDL into an atherogenic form *in vitro*. J Clin Invest, 1999. **103**(11): pp. 1547-60.

[9] Kensler, T.W., et al., Role of inflammatory cells in the metabolic activation of polycyclic aromatic hydrocarbons in mouse skin. Toxicol Appl Pharmacol, 1987. **90**(2): pp. 337-46.

[10] Saed, G.M., et al., Myeloperoxidase serves as a redox switch that regulates apoptosis in epithelial ovarian cancer. Gynecol Oncol, 2010. **116**(2): pp. 276-81.

[11] Galijasevic, S., et al., Myeloperoxidase interaction with peroxynitrite: chloride deficiency and heme depletion. Free Radic Biol Med, 2009. **47**(4): pp. 431-9.

[12] Maitra, D., et al., Melatonin can mediate its vascular protective effect by modulating free iron level by inhibiting hypochlorous acid-mediated hemoprotein heme destruction. Hypertension, 2011. **57**(5): p. e22; author reply e23.

[13] Maitra, D., et al., Reaction of hemoglobin with HOCl: mechanism of heme destruction and free iron release. Free Radic Biol Med, 2011. **51**(2): pp. 374-86.

[14] Maitra, D., et al., Mechanism of hypochlorous acid-mediated heme destruction and free iron release. Free Radic Biol Med, 2011. **51**(2): pp. 364-73.

[15] Muscara, M.N., et al., Wound collagen deposition in rats: effects of an NO-NSAID and a selective COX-2 inhibitor. Br J Pharmacol, 2000. **129**(4): pp. 681-6.

[16] Shi, H.P., et al., The role of iNOS in wound healing. Surgery, 2001. **130**(2): pp. 225-9.

[17] Witte, M.B. and A. Barbul, Role of nitric oxide in wound repair. Am J Surg, 2002. **183**(4): pp. 406-12.

[18] Sadrzadeh, S.M., et al., Hemoglobin. A biologic fenton reagent. J Biol Chem, 1984. **259**(23): pp. 14354-6.

[19] Sasaroli, D., Coukos, G. and N. Scholler, Beyond CA125: the coming of age of ovarian cancer biomarkers. Are we there yet? Biomark Med, 2009. **3**(3): pp. 275-288.

[20] Schummer, M., et al., Comparative hybridization of an array of 21,500 ovarian cDNAs for the discovery of genes overexpressed in ovarian carcinomas. Gene, 1999. **238**(2): pp. 375-85.

[21] Drapkin, R., et al., Human epididymis protein 4 (HE4) is a secreted glycoprotein that is overexpressed by serous and endometrioid ovarian carcinomas. Cancer Res, 2005. **65**(6): p. 2162-9.

[22] Gilks, C.B., et al., Distinction between serous tumors of low malignant potential and serous carcinomas based on global mRNA expression profiling. Gynecol Oncol, 2005. **96**(3): pp. 684-94.

[23] Hough, C.D., et al., Large-scale serial analysis of gene expression reveals genes differentially expressed in ovarian cancer. Cancer Res, 2000. **60**(22): pp. 6281-7.

[24] Galgano, M.T., Hampton, G.M. and H.F. Frierson, Jr., Comprehensive analysis of HE4 expression in normal and malignant human tissues. Mod Pathol, 2006. **19**(6): pp. 847-53.

[25] Bouchard, D., et al., Proteins with whey-acidic-protein motifs and cancer. Lancet Oncol, 2006. **7**(2): pp. 167-74.

[26] Rosen, D.G., et al., Potential markers that complement expression of CA125 in epithelial ovarian cancer. Gynecol Oncol, 2005. **99**(2): pp. 267-77.

[27] Scholler, N., et al., Bead-based ELISA for validation of ovarian cancer early detection markers. Clin Cancer Res, 2006. **12**(7 Pt 1): pp. 2117-24.

[28] Moore, R.G., et al., The use of multiple novel tumor biomarkers for the detection of ovarian carcinoma in patients with a pelvic mass. Gynecol Oncol, 2008. **108**(2): pp. 402-8.

[29] Kim, J.H., et al., Osteopontin as a potential diagnostic biomarker for ovarian cancer. JAMA, 2002. **287**(13): pp. 1671-9.

[30] Menon, U., et al., Prospective study using the risk of ovarian cancer algorithm to screen for ovarian cancer. J Clin Oncol, 2005. **23**(31): pp. 7919-26.

[31] Xu, F., et al., OVX1 as a marker for early stage endometrial carcinoma. Cancer, 1994. **73**(1): pp. 1855-8.

[32] Havrilesky, L.J., et al., Evaluation of biomarker panels for early stage ovarian cancer detection and monitoring for disease recurrence. Gynecol Oncol, 2008. **110**(3): pp. 374-82.

[33] Loria, V., et al., Myeloperoxidase: a new biomarker of inflammation in ischemic heart disease and acute coronary syndromes. Mediators Inflamm, 2008. **2008**: p. 135625.

[34] Dally, H., et al., Myeloperoxidase (MPO) genotype and lung cancer histologic types: the MPO -463 A allele is associated with reduced risk for small cell lung cancer in smokers. Int J Cancer, 2002. **102**(5): pp. 530-5.

[35] Piedrafita, F.J., et al., An Alu element in the myeloperoxidase promoter contains a composite SP1-thyroid hormone-retinoic acid response element. J Biol Chem, 1996. **271**(24): pp. 14412-20.

[36] Heinecke, J.W., et al., Tyrosyl radical generated by myeloper-

oxidase catalyzes the oxidative cross-linking of proteins. J Clin Invest, 1993. **91**(6): pp. 2866-72.

[37] Osterreicher, C.H., et al., Association of myeloperoxidase promotor polymorphism with cirrhosis in patients with hereditary hemochromatosis. J Hepatol, 2005. **42**(6): pp. 914-9.

[38] Choi, J.Y., et al., Iron intake, oxidative stress-related genes (MnSOD and MPO) and prostate cancer risk in CARET cohort. Carcinogenesis, 2008. **29**(5): pp. 964-70.

[39] Ekmekci, O.B., et al., Iron, nitric oxide, and myeloperoxidase in asthmatic patients. Biochemistry (Mosc), 2004. **69**(4): pp. 462-7.

[40] *Serum Iron*. Serum Iron 2006; Available from: http://uimc. discoveryhospital.com/main.php?id=1456.

[41] Medline, Serum Iron. Serum Iron 2010; Available from: http:// www.nlm.nih.gov/medlineplus/ency/article/003488.htm.

[42] Brennan, M.L., et al., Prognostic value of myeloperoxidase in patients with chest pain. N Engl J Med, 2003. **349**(17): pp. 1595-604.

[43] Baldus, S., et al., Myeloperoxidase serum levels predict risk in patients with acute coronary syndromes. Circulation, 2003. **108**(12): pp. 1440-5.

[44] Bozzini, C., et al., Biochemical and genetic markers of iron status and the risk of coronary artery disease: an angiography-based study. Clin Chem, 2002. **48**(4): pp. 622-8.

[45] de Valk, B. and J.J. Marx, Iron, atherosclerosis, and ischemic heart disease. Arch Intern Med, 1999. **159**(14): pp. 1542-8.

[46] Sullivan, J.L., Iron and the genetics of cardiovascular disease. Circulation, 1999. **100**(12): pp. 1260-3.

[47] Niederau, C., Iron overload and atherosclerosis. Hepatology, 2000. **32**(3): pp. 672-4.

[48] Zhu, C.S., et al., A framework for evaluating biomarkers for early detection: validation of biomarker panels for ovarian cancer. Cancer Prev Res (Phila), 2011. **4**(3): pp. 375-83.

[49] Baker, S.G., et al., Evaluating markers for the early detection of cancer: overview of study designs and methods. Clin Trials, 2006. **3**(1): pp. 43-56.

[50] Skates, S.J., et al., Toward an optimal algorithm for ovarian cancer screening with longitudinal tumor markers. Cancer, 1995. **76**(10 Suppl): pp. 2004-10.